PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12P 21/00, 21/02, C12N 5/00, 7/04, 15/00, 1/38. C12Q 1/66, 1/68/1/00

A1

(11) International Publication Number: WO 92/13063

(43) International Publication Date: 6 August 1992 (06.08.92)

(21) International Application Number: PCT/US92/00419

(22) International Filing Date: 17 January 1992 (17.01.92)

(30) Priority data:
644,292 18 January 1991 (18.01.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 644,292 (CIP)
Filed on 18 January 1991 (18.01.91)

(71) Applicant (*for all designated States except US*): ONCOGENE SCIENCE, INC. [US/US]; 106 Charles Lindburgh Blvd., Uniondale, NY 11553-3649 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*) : FOULKES, J., Gordon [US/US]; 35B East Rogues Path, Huntington Station, NY 11746 (US). CASE, Casey, C. [US/US]; 101 Charing Cross, Lynbrook, NY 11563 (US). LEICHTFRIED, Franz [AT/US]; 244-08 Jericho Turnpike, Bellerose, NY 11001 (US). PIELER, Christian [AT/US]; 27 Bedford Avenue, Westbury, NY 11590 (US). STEPHENSON, John [CA/US]; 315 Royal Seko, Santa Cruz, CA 95060 (US). MICHITCH, Richard [US/US]; 63 Fisher Road, Commack, NY 11275 (US).

(74) Agent: WHITE, John, P.; Cooper and Dunham, 30 Rockefeller Plaza, New York, NY 10112 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), NO, RU, SE (European patent), US.

Published
With international search report.

(54) Title: METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF GROWTH FACTOR GENES AND GROWTH FACTOR RECEPTOR GENES

(57) Abstract

The invention provides a method for effecting expression of growth factors and growth factor receptors in cells or in multicellular animals and methods for testing compounds as effectors of transcription of growth factors and growth factor receptors.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF GROWTH FACTOR GENES AND GROWTH FACTOR RECEPTOR GENES

This application is a continuation-in-part of U.S. Serial No. 644,292, filed January 18, 1991, the contents of which are hereby incorporated by reference into the present application.

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Growth Factors and Growth Factor Receptors

The proliferation and differentiation of normal cells is tightly controlled by exogenous growth factors. These polypeptides comprise a diverse group of regulatory agents that typically act in a hormone-like receptor-dependent manner (1) by initiating a cascade of responses involving a variety of key proteins. Some, but not all of these factors mediate their pleiotropic action by binding to and activating cell surface receptors with an intrinsic protein kinase activity (2). Growth factor receptors with protein tyrosine kinase activity have a similar molecular topology. All possess a large glycosylated, extracellular ligand (growth factor)

binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains the tyrosine kinase catalytic domain. This topology results in a receptor molecule with the ligand binding domain and the protein tyrosine kinase domain separated by the plasma membrane. Receptor activation due to extracellular ligand binding must therefore be translated across the membrane into activation of intracellular domain functions. Upon ligand binding to its receptor, a conformation change of the extracellular domain may occur, inducing receptor oligomerization which stabilizes the interaction between adjacent cytoplasmic domains and leads to activation of kinase function by molecular interactions (2). While exhibiting a similarity in structure, the tyrosine kinase receptors, by virtue of sequence similarity and distinct structural characteristics based on activation by receptor oligomerization, can be grouped into different subclasses.

Growth Factors

Within the past fifteen years, a large number of peptide growth factors have been identified. Many have been shown to exert potent stimulatory effects on cellular proliferation, while others with strong inhibitory activities have also been described (3). The actions exhibited by a specific peptide growth factor may be diverse; that is, it can have proliferative effects on one cell type while having anti-proliferative effects on a different cell type. Furthermore, the same growth factors can show effects unrelated to cellular proliferation. One example is epidermal growth factor (EGF), which stimulates the growth of keratinocytes and fibroblasts while inhibiting proliferations of hair

follicle cells and squamous carcinoma cells (4,5,6). EGF has also been shown to suppress gastric acid secretion (7). Growth factor action can also be influenced by the presence of other growth factors, suggesting an interaction between peptides in regulating growth factor activity. For example, transforming growth factor-β stimulates the in vitro growth of fibroblasts in the presence of platelet-derived growth factor (PDGF), while inhibiting their growth in the presence of epidermal growth factor (EGF). It appears, therefore, that specific peptides are not limited to a single physiological activity, but form part of a complex cellular signalling pathway.

As stated previously, a large number of growth factors have been described. Identification and description of each of these factors individually is well beyond the scope of this brief introduction. However, listed below is a limited number of growth factors to serve as examples.

1. Transforming growth factor-β (TGF-β): Thus far, at least 4 closely related members of the TGF-β family have been described (8,9,10,11). Biological activities described for TGF-β include stimulating growth of cells in soft agar (12) as well as stimulating mesenchymal cell growth in monolayer (13). TGF-β is also chemotactic for fibroblasts and macrophages (14,15) and was shown to have a pronounced effect an extracellular matrix production by causing an increase in collagen, fibronectin and proto-glycan expression (16,17). It has also been shown to be a potent inhibitor of a variety of cell types, including epithelial, endothelial, lymphoid and myeloid cells (18,19,20,68).

2. Hemopoietic Colony Stimulatory Factors (CSFs): These glycoprotein growth factors are essential for the survival, growth and differentiation of hemopoietic progenitor cells, in vitro. At least five different classes of CSF have been described: M-CSF (macrophage), GM-CSF (granulocyte/macrophage), G-CSF (granulocyte) multi-CSF or IL-3 (erythroid, megakaryocyte and eosinophil) and stem cell factor, or c-kit ligand. These have been defined by their ability to stimulate colony formation of their respective cell type in semi-solid cultures of bone marrow cells (21).

3. Stem Cell Growth Factors (SCGFs): This group of growth factors consists of these separate members: SCGF-1, which stimulates the proliferation of the pluripotent cells that produce it; SCGF-2, which stimulates fibroblast growth and induces them to express properties of the transformed phenotype; SCGF-3, which stimulates the proliferation of friend erythroleukemia cells and inhibits their induced differentiation (22).

4. Growth Hormone (GH): GH has been implicated in major developmental and basic metabolic regulation pathways of the body. Its production is stimulated at the level of gene transcription by growth hormone releasing factor (GRF). In 3T3 cells, it was demonstrated that growth hormone induced adipose differentiation (23).

5. Interleukins: The interleukins are a family of protein hormones which regulate the growth, differentiation and activities of leukocytes. Representative members of this family include

interleukin 2 (IL-2) which mediates the growth and activation of B and T cells, interleukin 3 (IL-3) which has hematopoietic growth factor activity, and IL-5 which is also known as eosinophile differentiation factor.

Growth Factor Receptors

Typically, growth factors bind to their cognate receptor and thereby induce conformational changes in the receptor which in turn lead to activation of kinase activity (in receptor tyrosine kinase family members) that trigger of an array of cellular responses, including Na+/H+ exchanges, Ca^{2+} influx, activation of phospholipase C, and stimulation of glucose and amino acid transport (2). A number tyrosine kinase receptors of potential biological importance have been identified. Representative examples include:

1. c-erbB2: this oncogene encoded product as a 185,000 kd transmembrane glycoprotein with tyrosine kinase activity which shares sequence similarity to the epidermal growth factor receptor (EGF-R) (24). Originally identified in ethylnitrosourea-induced rat neuroblastomas (25), the erbB-2 gene has subsequently been shown to be amplified in several adenocarcinomas and is overexpressed in about 30% of human breast cancer patients (26). p185erbB-2 was shown to be necessary for maintenance of the malignant phenotype of cells transformed by erbB-2 (27), implicating the protein in tumorigenesis. Recently, a 30 kD glycoprotein (gp 30) has been described which interacts with erbB-2 and may, in fact, be the ligand for p185erbB-2 (28).

2. Platelet Derived Growth Factor Receptors: PDGF trigger proliferation and chemotaxis by stimulating the tyrosine kinase activity of the PDGF receptors. Two receptor types exist, called α and β. Each responds differently to the various combinations of PDGF dimers (AA, AB and BB)(68).

3. c-kit: a member of the PDGF receptor subfamily and the gene product of the murine white spotting (W) locus, c-kit encodes a transmembrane tyrosine kinase receptor (29,30). The c-kit/W gene functions in immature progenitor cell populations and in more mature cell types of the three cell lineages. Recently, the ligand for the c-kit proto-oncogene receptors has been identified as the gene product of the steel (Sl) locus of the mouse (31,32).

Some growth factor receptors (e.g. the erythropoietin receptor) do not appear to possess intrinsic tyrosine kinase activity and must transduce ligand binding signals by other means.

The proliferation and differentiation of normal cells is tightly controlled by growth factors. However, a variety of structural alterations in receptor-derived oncogene products or else overexpression or inappropriate expression of a specific growth factor or inhibition of the expression of a factor whose function is to inhibit cell growth may release the cell from its normal growth constraints and result in a transformed cell. Constitutive activation of receptor tyrosine kinase signalling function can be the result of deletion of the extracellular binding domain, thereby eliminating the negative control that the structure normally exerts on the cytoplasmic domains (as in v-erbB and v-kit). Point

mutations within the extracellular domain can lead to intracellular activation, as in v-*fms*. Overexpression of c-*erb*B2 is often involved in breast, stomach and ovarian cancers, while overexpression of the EGF receptor gene has been observed in breast, bladder, brain and lung tumors.

Clearly, a number of diseases are associated with inappropriate expression of growth factor or growth factor receptor genes. Pharmaceuticals which restore the proper levels of expression would obviously be of use for the treatment of these diseases.

Additionally, there are a number of clinical situations where a non-physiological increase or decrease of the normal levels of growth factors or growth factor receptors would be useful; the over expression of growth hormone to counteract the degenerative effects of ageing or an increase in hematopoietic stem cell factor to bolster immunity are two possible examples.

Traditional growth factor-based clinical approaches have been to supply the growth factors via injection of purified protein, or to seek classic receptor agonists. In spite of their great potential, protein based pharmaceuticals suffer from several general limitations; they need to be delivered by injection, are unstable (affecting shelf-life) and they are very expensive to manufacture. Here we describe a method to find small molecular weight organic compounds, which have some of the same biological consequences as growth factors, or to modulate the level of growth factor receptors. The general approach is to screen compound libraries or natural products extracts for substances which increase or decrease expression of the endogenous growth factor or

growth factor receptor genes.

The expression of a specific gene can be regulated at any step in the process of producing an active protein. Modulation of total protein activity may occur via transcriptional, transcript-processing, translational or post-translational mechanisms. Transcription may be modulated by altering the rate of transcriptional initiation or the progression of RNA polymerase (33). Transcript-processing may be influenced by circumstances such as the pattern of RNA splicing, the rate of mRNA transport to the cytoplasm or mRNA stability. This invention concerns the use of molecules which act by modulating the <u>in vivo</u> concentration of their target proteins via regulating gene transcription. The functional properties of these chemicals are distinct from previously described molecules which also affect gene transcription.

Researchers have documented the regulation of transcription in bacteria by low molecular weight chemicals (34,35). Extracellular xenobiotics, amino acids and sugars have been reported to interact directly with an intracellular proteinaceous transcriptional activator or repressor to affect the transcription of specific genes.

Transcriptional regulation is sufficiently different between procaryotic and eucaryotic organisms so that a direct comparison cannot readily be made. For example, procaryotic cells lack a distinct membrane bound nuclear compartment. Furthermore, the structure and organization of procaryotic DNA elements responsible for initiation of transcription differ markedly from those of eucaryotic cells.

The eucaryotic transcriptional unit is much more complex than its procaryotic counterpart and consists of additional elements which are not commonly found in bacteria, including enhancers and other cis-acting DNA sequences (36,37). Procaryotic transcription factors most commonly exhibit a "helix-turn-helix" motif in the DNA binding domain of the protein (38,39). Eucaryotic transcriptional factors frequently contain a "zinc finger" (39,40),a "leucine zipper" (41), a "helix-loop-helix" or "helix-turn-helix" motif (42). Furthermore, several critical mechanisms at the post-transcriptional level such as RNA splicing and polyadenylation are typically not found in procaryotic systems (43,44).

In higher eucaryotes, modulation of gene transcription in response to extracellular factors can be regulated in both a temporal and tissue specific manner (45). For example, extracellular factors can exert their effects by directly or indirectly activating or inhibiting tissue specific transcription factors (45,33).

Modulators of transcription factors involved in direct regulation of gene expression have been described, and include those extracellular chemicals entering the cell passively and binding with high affinity to their receptor-transcription factors. This class of direct transcriptional modulators include steroid hormones and their analogs, thyroid hormones, retinoic acid, vitamin D_3 and its derivatives, and dioxins, a chemical family of polycyclic aromatic hydrocarbons (40,46,47).

Dioxins are molecules generally known to modulate transcription, however, dioxins bind to naturally-occurring receptors which respond normally to xenobiotic agents via transcriptionally activating the

expression of cytochrome P450. Similarly, plants also have naturally occurring receptors to xenobiotics to induce defense pathways. For example, the fungal pathogen Phytophthora megasperma induces an anti-fungal compound in soybeans. Such molecules which bind to the defined ligand binding domains of such naturally occurring receptors are not included on the scope of this invention.

The clinical use of steroid hormones, thyroid hormones, vitamin D_3 and their analogs demonstrates that agents which modulate gene transcription can be used for beneficial effects, although these agents can exhibit significant adverse side effects. Obviously, analogs of these agents could have similar clinical utility as their naturally occurring counterparts by binding to the same ligand binding domain of such receptors. These types of molecules do not fall within the scope of this invention because they function by binding to the ligand-binding domain of a receptor normally associated with a defined physiological effect.

Indirect transcriptional regulation involves one or more general signal transduction mechanisms. This type of regulation typically involves interaction with a receptor, the receptor being part of a multistep intracellular signaling pathway, the pathway ultimately modulating the activity of nuclear transcription factors. This class of indirect transcriptional modulators include polypeptide growth factors such as platelet-derived growth factor, epidermal growth factor, cyclic nucleotide analogs, and mitogenic tumor promoters such as PMA (48,49,50).

It is well documented that a large number of chemicals,

both organic and inorganic, e.g. metal ions, can non-specifically modulate transcription. Most heavy metals modulate gene expression through receptors in a mechanism similar to that employed by dioxin, steroid hormones, vitamin D3 and retinoic acid.

Researchers have used nucleotide analogs in methods to non-specifically modulate transcription. The mechanism involves incorporating nucleotide analogs into nascent mRNA or non-specifically blocking mRNA synthesis. Similarly, researchers have used alkylating agents, e.g. cyclophosphamide, or intercalating agents, e.g. doxorubicin, to non-specifically inhibit transcription.

Moreover, chemical inhibitors of hydroxymethyl-glutaryl CoA reductase, e.g. lovastatin, are known to indirectly modulate transcription by increasing expression of hepatic low density lipoprotein receptors as a consequence of lowered cholesterol levels.

Signal effector type molecules such as cyclic AMP, diacylglycerol, and their analogs are known to non-specifically regulate transcription by acting as part of a multistep protein kinase cascade reaction. These signal effector type molecules bind to domains on proteins which are thus subject to normal physiological regulation by low molecular weight ligands (51,52).

The specific use of sterol regulatory elements from the LDL receptor gene to control expression of a reporter gene has recently been documented in PCT/US88/10095. One aspect of PCT/US88/10095 deals with the use of specific sterol regulatory elements coupled to a reporter as a means to screen for drugs capable of stimulating cells to synthesize the LDL receptor. PCT/US88/10095 describes

neither the concept of simultaneously screening large numbers of chemicals against multiple target genes nor the existence of transcriptional modulators which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the growth factor or growth factor receptor genes, and (c) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a defined ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect. The main focus of PCT/US88/10095 is the use of the sterol regulatory elements from the LDL receptor as a means to inhibit expression of toxic recombinant biologicals.

The use of molecules to specifically modulate transcription of a growth factor or growth factor receptor gene as described herein has not previously been reported and its use will bring surprise since available literature does not propose the use of a molecule, as described, in a method to specifically modulate transcription. Instead, the available literature has reported methods which define domains of transcriptional regulating elements of a growth factor and growth factor receptor genes.

Further, the practice of using a reporter gene to analyze nucleotide sequences which regulate transcription of a gene-of-interest is well documented. The demonstrated utility of a reporter gene is in its ability to define domains of transcriptional regulatory elements of a gene-of-interest. Reporter genes which express proteins, e.g. luciferase, are widely utilized in such studies. Luciferases expressed by the North American firefly,

Photinus pyralis and the bacterium, *Vibrio fischeri* were first described as transcriptional reporters in 1985 (53,54). Reporter genes have not been previously used to identify compounds which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene encoding growth factors or growth factor receptors, and (c) binds to DNA or RNA, or bind to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

A method to define domains of transcriptional regulating elements of a gene-of-interest typically has also involved use of phorbol esters, cyclic nucleotide analogs, concanavalin A, or steroids, molecules which are commonly known as transcriptional modulators. However, available literature shows that researchers have not considered using a transcription screen to identify specific transcriptional modulators. Apparently, success would be unlikely in doing so, however, we have demonstrated herein that this is not the case.

There is utility in developing the method of transcriptional modulation of growth factor and growth factor receptor genes by using such molecules as described herein. This method will allow the development of novel pharmaceuticals and circumvent many of the problems associated with the therapeutic use of recombinant biological factors.

Problems associated with the therapeutic use of recombinant biological factors include the technical difficulties of large scale protein purification, the

high costs of protein production, the limited shelf-life of most proteins and in some cases a short biological half-life of the administered protein in the organism. Additionally, therapeutic delivery of proteins normally requires injection. The method described herein provides a means of up-regulating the expression of proteins which are not readily amenable to administration as injectable biologicals.

Furthermore, molecules specifically regulating the activity of one member of a group of closely related proteins are difficult to identify. Molecules, structurally related at the protein level, may possess distinct regulatory elements at the DNA level which control their expression. Thus, molecules such as the chemical transcriptional modulators defined herein can provide a greater opportunity for specifically modulating the activity of structurally related proteins.

Finally, the molecules described herein may also serve to mimic normal physiological response mechanisms, typically involving the coordinated expression of one or more groups of functionally related genes. Therefore, determining whether a molecule can specifically transcriptionally modulate the expression of a growth factor or growth factor receptor gene and the ultimate clinical use of the molecule provides a therapeutic advantage over the use of single recombinant biologicals, or drugs which bind directly to the final target protein encoded by the gene-of-interest.

Summary of the Invention

The invention provides a method for directly transcriptionally modulating the expression of a gene encoding a growth factor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the growth factor encoded by the gene which is expressed by the cell. In this method the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene encoding the growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

The invention also provides a method for directly transcriptionally modulating the expression of a gene encoding a growth factor receptor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the growth factor receptor encoded by the gene which is expressed by the cell. In this method the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene

encoding the growth factor receptor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

This invention further provides for a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each such cell comprises DNA which consists essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the growth factor, and (iii) a DNA sequence encoding a polypeptide other than the growth factor, which polypeptide is capable of producing a detectable signal. The DNA sequence is coupled to, and under the control of, the promoter, and the contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable detectable signal to be produced by the polypeptide so expressed. This allows for a quantitative determination of the amount of the signal produced. By comparing the amount of detectable signal produced with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, this method allows one to identify the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identifying the molecule as a molecule capable of

transcriptionally modulating the expression of the gene encoding the growth factor.

The invention still further provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the gene encoding the growth factor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable change in the amount of the polypeptide produced, and quantitatively determining the amount of the polypeptide produced. By comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule, the molecule is identified as one which causes a change in the amount of polypeptide expressed, and thus identified as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

The invention further encompasses a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor. This method comprises contacting a sample which

contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each of the cells so contacted comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of gene encoding the growth factor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter. The contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. The amount of the mRNA produced is quantitatively determined and the amount so determined compared with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to identify the molecule as one which causes a change in the detectable mRNA amount of, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

This invention further provides for a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each such cell comprises DNA which consists essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of the growth factor receptor, and (iii) a DNA sequence encoding a polypeptide other than the growth factor receptor, which polypeptide

is capable of producing a detectable signal. The DNA sequence is coupled to, and under the control of, the promoter, and the contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor receptor, causes a measurable detectable signal to be produced by the polypeptide so expressed. This allows for a quantitative determination of the amount of the signal produced. By comparing the amount of detectable signal produced with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, this method allows one to identify the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

The invention still further provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of the gene encoding the growth factor receptor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor

receptor, causes a measurable change in the amount of the polypeptide produced, and quantitatively determining the amount of the polypeptide produced. By comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule, the molecule is identified as one which causes a change in the amount of polypeptide expressed, and thus identified as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

The invention further encompasses a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each of the cells so contacted comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of gene encoding the growth factor receptor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter. The contacting is effected under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor receptor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. The amount of the mRNA produced is quantitatively determined and the amount so determined compared with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to identify the molecule as one which

causes a change in the detectable mRNA amount of, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

A screening method is also provided. This screening method comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested.

Also disclosed is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding growth factors or receptors which comprises essentially simultaneously screening the molecules against the growth factors or receptors according to the methods mentioned above.

A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding an growth factor, the expression of which is associated with a defined physiological or pathological effect in the organism, is also included. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect, which molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene encoding the growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to

which ligand-binding domain is normally associated with a defined physiological or pathological effect.

A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding an growth factor receptor, the expression of which is associated with a defined physiological or pathological effect in the organism, is also included. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect, which molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene encoding the growth factor receptor, and (c)binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

Brief Description of the Figures

Figure 1 is a view of the mammalian expression shuttle vector pUV102 with its features. The mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions and the insertion of a neomycin resistance gene coupled to the herpes simplex virus thymidine kinase promoter (TK-NEO).

Figure 2 is a partial restriction enzyme cleavage map of the plasmid pD0432 which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 3 is a partial restriction enzyme cleavage map of the plasmid pSVLuci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 4 is a partial restriction enzyme cleavage map of the plasmid pMLuci which contains the luciferase gene of the firefly, Photinus pyralis and the mouse mammary tumor virus long terminal repeat.

Figure 5 provides the nucleotide sequences of six oligonucletides, pUV-1 through pUV-6, which were annealed, ligated, and inserted into the SalI/EcoR1 sites of the plasmid pTZ18R.

Figure 6 is a diagrammatic representation of the construction of the plasmid pUV001 from the plasmids pTZ18R and pBluescript KS(+).

Figure 7 is a diagrammatic representation of the construction of the plasmid pUV100 from the plasmid pUV001 and two DNA fragments, the XbaI/XmaI fragment from

pMLuci and the XmaI/BamHI fragment from pMSG.

Figure 8 is a diagrammatic representation of the construction of the plasmid pUV100-3 from the plasmid pUV100 and a 476 b fragment containing a dimeric SV40 polyadenylation site.

Figure 9 is a diagrammatic representation of the construction of the plasmids pUV102 and pUV103 from the plasmid pUV100-3 and D-link oligonucleotides and the plasmid pUV100-3 and R-link oligonucleotides, respectively.

Figure 10 provides the nucleotide sequences of oligos 1-4 used for the construction of a synthetic HSV-Thymidine Kinase promoter and provides a diagrammatic representation of the HSV-TK promoter.

Figure 11 is a diagrammatic representation of the construction of the plasmid pTKL100 which contains the luciferase gene from the firefly, Photinus pyralis and the HSV-TK promoter sequence.

Figure 12 is a diagrammatic representation of the construction of the plasmid pTKNEO which contains the neo gene, from about 3.5 kb NheI/XmaI fragment from pTKL100, and the about 0.9 kb BstBI/BglII fragment containing the neo coding region from pRSVNEO.

Figure 13 is a diagrammatic representation of the construction of the plasmid pTKNEO2 from the plasmid pTKNEO and the oligonucleotides Neo 1 and 2.

Figure 14 is a diagrammatic representation of the construction of the plasmid pTKNEO3 from the plasmid

PTKNEO2 and about 0.9 kb EcoRl/SalI fragment from pMC1NEO.

Figure 15 is a partial restriction map of plasmid pUXLuci, a vector used in the construction of the human growth hormone reporter vector.

Figure 16 is a partial restriction enzyme cleavage map of the plasmid phGH:CAT which contains the CAT gene and human growth hormone promoter sequences.

Figure 17 is a partial restriction enzyme cleavage map of the plasmid phGH-Luci which contains the luciferase gene from the firefly, Photinus pyralis and human growth hormone promoter sequences.

Figure 18 is a partial restriction enzyme cleavage map of the plasmid pNEU106 which contains neu upstream sequences fused to the luciferase coding region.

Figure 19 is a partial restriction enzyme cleavage map of the plasmid pKRAS106 which contains K-ras upstream sequences fused to the luciferase gene from the firefly, Photinus pyralis.

Figure 20 is a graphical representation of the decay of reporter gene signal after treatment of cells with Actinomycin D. Plotted is relative intensity of the signal versus time after ActD addition.

Figure 21 is a quality assurance analysis of a high throughput screen measuring the ratios of negative values at various positions within a plate. The expected value is 1.0.

Figure 22 is a quality assurance analysis of a high throughput screen measuring a robustified coefficient of variance for the negative controls on a number of plates. Values less than 10 are acceptable.

Figure 23 is a quality assurance analysis of a high throughput screen measuring a robustified coefficient of variance for the positive controls on a number of plates. Values less than 10 are acceptable.

Figure 24 is a quality assurance analysis of a high throughput screen measuring a response of a reporter cell line to three different concentrations of a compound known to induce transcription.

Detailed Description of the Invention

As used in this application, the following words or phrases have the meanings specified.

Antisense nucleic acid means an RNA or DNA molecule or a chemically modified RNA or DNA molecule which is complementary to a sequence present within an RNA transcript of a gene.

Directly transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of the gene through the binding of a molecule to (1) the gene (2) an RNA transcript of the gene, or (3) a protein which binds to (i) such gene or RNA transcripts, or (ii) a protein which binds to such gene or RNA transcript.

A gene means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

Growth factor means a polypeptide factor, either soluble or displayed on the external surface of a plasma membrane, upon binding to a specific growth factor receptor on the surface of the appropriate cell type, stimulates the growth, division or differentiation of the cell. Growth factors may exhibit diverse effects (or no effects) on other cell types.

Growth factor receptor means a membrane spanning polypeptide which, when present on the surface of the appropriate cell type, and upon the binding of a specific growth factor, initiates a physiological response, such

as growth, division or differentiation.

Indirectly transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of such gene through the action of a molecule which cause enzymatic modification of a protein which binds to (1) the gene or (2) an RNA transcript of the gene, or (3) protein which binds to (i) the gene or (ii) an RNA transcript of the gene. For example, altering the activity of a kinase which subsequently phosphorylates and alters the activity of a transcription factor constitutes indirect transcript modulation.

Ligand means a molecule with a molecular weight of less than 5,000, which binds to a transcription factor for a gene. The binding of the ligand to the transcription factor transcriptionally modulates the expression of the gene.

Ligand binding domain of a transcription factor means the site on the transcription factor at which the ligand binds.

Modulatable transcriptional regulatory sequence of a gene means a nucleic acid sequence within the gene to which a transcription factor binds so as to transcriptionally modulate the expression of the gene capable of regulating the transcription of a hematopoietic gene including, but not limited to, promoters, enhancers, attenuators, and silencers.

Receptor means a transcription factor containing a ligand binding domain.

Specifically transcriptionally modulate the expression of

a gene means to transcriptionally modulate the expression of such gene alone, or together with a limited number of other genes.

Transcription means a cellular process involving the interaction of an RNA polymerase with a gene which directs the expression as RNA of the structural information present in the coding sequences of the gene. The process includes, but is not limited to the following steps: (1) the transcription initiation, (2) transcript elongation, (3) transcript splicing, (4) transcript capping, (5) transcript termination, (6) transcript polyadenylation, (7) nuclear export of the transcript, (8) transcript editing, and (9) stabilizing the transcript.

Transcription factor for a gene means a cytoplasmic or nuclear protein which binds to (1) such gene, (2) an RNA transcript of such gene, or (3) a protein which binds to (i) such gene or such RNA transcript or (ii) a protein which binds to such gene or such RNA transcript, so as to thereby transcriptionally modulate expression of the gene.

Transcriptionally modulate the expression of a gene means to change the rate of transcription of such gene.

Triple helix means a helical structure resulting from the binding of one or more oligonucleotide to double stranded DNA.

The invention also provides a method for directly transcriptionally modulating the expression of a gene encoding a growth factor, the expression of which is associated with a defined physiological or pathological

effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the growth factor encoded by the gene which is expressed by the cell. In this method the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene encoding the growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

The invention also provides a method for directly transcriptionally modulating the expression of a gene encoding a growth factor receptor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. This method comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the growth factor receptor encoded by the gene which is expressed by the cell. In this method the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene encoding the growth factor receptor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

In a preferred embodiment, the molecule does not naturally occur in any cell, whether of a multicellular or a unicellular organism. Alternatively, the molecule is naturally occurring, but not normally found in the cell. In a presently more preferred embodiment, the molecule is not a naturally occurring molecule, e.g. is a chemically synthesized entity. The cell may be a cell of the multicellular organism, which could included, a fish cell, a avian cell, an animal cell, human cell, bovine cell, or a porcine cell.

The transcriptional modulation in the method mentioned above may comprises upregulation or downregulation of expression of the gene encoding the growth factor or receptor. Additionally it may bind to a modulatable transcriptional sequence of the gene.

Additionally the molecule may an antisense nucleic acid, double-stranded nucleic acid, a nucleic acid capable of forming a triple helix with double-stranded DNA,

The growth factor in the above methods may be human growth factor, bovine growth factor, the porcine growth factor, a fish growth factor, an avian growth factor.

The growth factor may be a transforming growth factor beta, an epidermal growth factor, a transforming growth factor alpha, platelet derived growth factor, vascular endothelial cell growth factor, fibroblast growth factor, nerve growth factor, a bone morphogenic protein, an insulin, an insulin-like growth factor, an interleukin such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 or a hematopoietic growth factor such as G-CSF, GM-CSF, EPO, IL-3, M-CSF, c-Kit ligand.

The growth factor receptor may be a human growth factor receptor, a bovine growth factor receptor, a porcine growth factor receptor, a fish growth factor receptor, or an avian growth factor receptor.

The growth factor receptor may be the receptor for a transforming growth factor ß, an epidermal growth factor, a transforming growth factor α. In addition the growth factor receptor may be erbB2 (neu), platelet derived growth factor receptor, VEGF receptor, FGF receptor, NGF receptor, an interleukin receptor such as IL-I receptor, IL-2 receptor, IL-2α receptor, IL-3 receptor, a hematopoietic growth factor such as G-CSF receptor, GM-CSF receptor, EPO receptor, C-fms (M-CSF receptor), or c-Kit (Kit ligand receptor) or an insulin receptor.

The invention further includes a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. The cell used contains DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the gene encoding the growth factor, and (iii) a DNA sequence encoding a polypeptide other than the growth factor, which polypeptide is capable of producing a detectable signal, which DNA sequence is coupled to, and under the control of, the promoter. The method is carried out under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable detectable signal to be produced by the polypeptide so

expressed. This aloes on to quantitatively determining the amount of the signal produced by comparing the amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule. Thus one may identifying the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

Further disclosed is a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. The cell used contain DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the gene encoding the growth factor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter This is carried out under such conditions that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable change in the amount of the polypeptide produced. This aloes one to quantitatively determine the amount of the polypeptide so produced, comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule. Thus one is able to identify the molecule as one which causes a change in the amount of the polypeptide expressed, and thus identifying the molecule

as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

The invention also provides for a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor. This method which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the gene encoding the growth factor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of the promoter. This is carried out under such conditions that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. This aloes one to quantitatively determine the amount of the mRNA produced. Thus by comparing the amount so determined with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, one may identify the molecule as one which causes a change in the detectable mRNA amount of. One may thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor, is also disclosed in the invention. This method comprises

contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. The cells contain DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of the gene encoding the growth factor receptor, and (iii) a DNA sequence encoding a polypeptide other than the growth factor receptor, which polypeptide is capable of producing a detectable signal, which DNA sequence is coupled to, and under the control of, the promoter. This method is carried out under such conditions that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor receptor, causes a measurable detectable signal to be produced by the polypeptide so expressed. This aloes one to quantitatively determine the amount of the signal produced, comparing the amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule. Thus one is able to identify the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

The invention includes a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. The cell used contains DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the

gene encoding the growth factor receptor, (ii) a promoter of the gene encoding the growth factor receptor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter. This method is carried out under such conditions that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor receptor, causes a measurable change in the amount of the polypeptide produced. This allows one to quantitatively determine the amount of the polypeptide so produced, by comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identify the molecule as one which causes a change in the amount of the polypeptide expressed. This allows one to identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

Further provided is a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor. This method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. The cell used contain DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of the gene encoding the growth factor receptor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter. This is carried out under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene

encoding the growth factor receptor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence. From this one may quantitatively determine the amount of the mRNA produced, by comparing the amount so determined with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule. Thus one may identify the molecule as one which causes a change in the detectable mRNA amount of, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

The sample may comprises cells in monolayers, or in suspension. The cells may comprise animal cells, which may include human cells, bovine cells, murine cells, porcine cells, fish cells, or avian cells.

The predefined number of cells may be from about 1 to about 5 X 10^5 cells, or from about 2 X 10^2 to about 5 X 10^4 cells. The predetermined amount of the molecule to be tested may be based upon the volume of the sample, or from about 1.0 pM to about 20 μM, or from about 10 nM to about 500 μM. The contacting may be effected from about 1 to about 24 hours, or about 2 to about 12 hours.

The contacting may be effected with more than one predetermined amount of the molecule to be tested. The molecule to be tested may be a purified molecule.

The modulatable transcriptional regulatory sequence may comprises a cloned genomic regulatory sequence. The DNA may consists essentially of more than one modulatable transcriptional regulatory sequence. The DNA sequence encoding the polypeptide may be inserted downstream of

the promoter of the gene encoding a growth factor or receptor by homologous recombination.

The invention includes the case where the polypeptide is a luciferase, chloramphenicol acetyltransferase, β glucuronidase, β galactosidase, neomycin phosphotransferase, alkaline phosphatase or guanine xanthine phosphoribosyltransferase. Additionally the polypeptide may be capable of complexing with an antibody or biotin. Further, the mRNA may be detected by quantitative polymerase chain reaction.

Further provided for in the invention is a screening method according to the methods above which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested. The plurality of samples may comprise more that about 10^4 samples, or more than about 5×10^4 samples.

Also included in the disclosure is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding growth factors or receptors according to the methods of above.

Further provided for is a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding growth factor receptor(s). This method comprises essentially simultaneously screening the molecules against the genes encoding the

growth factor receptor(s) according to the method of above. This method may have more than about 10^3 samples per week contacted with different molecules.

Pursuant to the provisions of the Budapest Treaty on the International Recognition of Deposit of Microoganisms for Purpose of Patent Procedure, the plasmid and cell lines listed below have been deposited with the American Type Culture Collection ("ATCC"), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.:

1. a plasmid designated pUV106, deposited under ATCC Accession No. 40946;

2. a human colon adenocarcinoma cell line, transfected with pHRA521, designated H21, deposited under ATCC Accession No. CRL 10640;

3. a HTB-30 human colon adenocarcinoma cell line, transfected with pNEU106, designated N-2, deposited under ATCC Accession No. CRL 10658;

4. a SW 480 human breast carcinoma cell line, transfected with pKRAS106, designated K-2, deposited under ATCC Accession No. CRL 10662;

5. a NIH Swiss mouse embryo cell line, NIH 3T3, transfected with the MMTV reporter plasmid, designated M10, deposited under ATCC Accession No. CRL 10659; and

6. a GC rat pituitary cell line, transfected with the growth hormone reporter plasmid, designated 532, deposited under ATCC Accession No. CRL 10663.

A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding an growth factor, the expression of which is associated with a defined physiological or pathological effect in the organism, is also included. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. In this the method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene encoding the growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding an growth factor receptor, the expression of which is associated with a defined physiological or pathological effect in the organism, is also included. This method comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. In this method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene encoding the growth factor receptor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally

associated with a defined physiological or pathological effect.

The molecule in the above methods may be an antisense, nucleic acid, a double stranded nucleic acid molecule, or a nucleic acid capable of forming a triple helix with double stranded DNA.

The above method additionally include the case where the multicellular organism is a human being, an animal, which may include a cow, a pig, a fish, a chicken, or a mouse.

The defined pathological effect may be a disorder where modulated expression of the gene encoding a growth factor is associated with amelioration of the disorder. The defined pathological effect may be pituitary dwarfism, acute catabolic trauma, obesity, or the combined degenerative disorders of old age. In addition the defined pathological effect may be a disorder where modulated expression of the gene encoding a growth factor receptor is associated with amelioration of the disorder. The defined pathological effect may be bladder cancer, brain cancer, breast cancer, colon cancer, lung cancer or ovarian cancer.

The administering in the above methods may be by topical contact, or by oral, transdermal, intravenous, intramuscular or subcutaneous administration.

This invention is illustrated in the Experimental Detail section which follow. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

A. Cell Culture

All media and reagents used for routine cell culture were purchased from Gibco (Grand Island, NY), Hazelton (Lenexa, KS), or Whittaker M.A. Biologicals (Walkersville, MD). Fetal calf serum (FCS) was from Hyclone (Logan, UT), and nutrients used for serum-free defined media were purchased from Sigma (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), Bachem (Torrance, CA) and Collaborative Research (Bedford, MA). (55,56).

A rat pituitary cell line, designated GC, used for transfection of plasmids containing the human growth hormone promoter, is maintained in DMEM and Ham's F12 medium (1:1), supplemented with 12.5% FCS. For HTP screening, transfected GC clones will be transferred to serum free defined medium consisting of DMEM and Ham's F12 medium (1:1) supplemented with growth factors, hormones and nutrients as described previously.

A human breast adenocarcinoma derived cell line, SK-BR-3 (ATCC HTB 30) was used for the experiments concerning expression of the neu (ErbB2) proto-oncogene. This cell line was maintained on DMEM, 15% FCS and 1 ug/ml insulin. Stable transfectants of this cell line were selected in this same medium with the addition of G418 to a final concentration of 0.4 mg/ml.

A human colon adenocarcinoma cell line, SW480 (ATCC CCL 228) was used for experiments concerning expression of

the K-ras proto-oncogene (as a control for specificity). This cell line was maintained on DMEM, 15% fetal calf serum (FCS), 1% Nonessential amino acids (NEAA). Stable transfectants of this cell line were selected in the same medium with the addition of G418 (Geneticin, Gibco) to a final concentration of 0.6 mg/ml.

A murine embryonic fibroblast cell line, NIH 3T3 (ATCC# CCL92), was used for the transfection of plasmids carrying the MMTV promoter. These cells were maintained on DMEM, supplemented with 10% FCS.

B. Construction of the Luciferase-Fusion Reporter Vector

Unless otherwise indicated, molecular cloning procedures were performed essentially according to Maniatis et al. (57). Oligonucleotides were synthesized by the beta-cyanoethyl phosphoramidite method according to protocols provided by the manufacturer of the DNA-synthesizer (Model 380A, Applied Biosystems (Foster City, CA).

A mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions to be used in high-throughput screens to identify transcriptionally modulating chemicals. Features of the plasmid are shown in Figure 1. The shuttle vector was constructed in several steps.

The firefly luciferase gene was removed from the plant expression plasmid pDO432 (58) (Figure 2) as a 1.9 kb BamHI fragment and cloned into the BamHI site of pSVL (Pharmacia, Piscataway, NJ), a mammalian expression vector containing the SV40 promoter. The resulting

plasmid (pSVLuci; Figure 3) was digested with XhoI and SalI to produce a 2.4 kb fragment containing the luciferase coding sequences and the SV40 late polyadenylation site. This fragment was inserted into the XhoI site of pMSG (Pharmacia, Piscataway, NJ), a eucaryotic expression vector containing the MMTV promoter. The resulting MMTV promoter-luciferase fusion plasmid (pMLuci; Figure 4) was used to transfect NIH/3T3 cells as described below. Similar constructs can be made using luciferase vectors from Clontech (Palo Alto, CA).

Six oligonucleotides (pUV-1 through pUV-6) (SEQ ID NO: 1-6) were synthesized (see Figure 5 for sequence) The sequences of pUV-1, pUV-2 and pUV-3 correspond to a multicloning site, the beta-globin leader sequence and the first 53 bases of the firefly luciferase coding region. The sequences of pUV-4, pUV-5 and pUV-6 are complementary to the first three oligonucleotides. The pUV oligonucleotides were annealed, ligated and inserted into the SalI/EcoRI sites of pTZ18R (Pharmacia, Piscataway NJ) (Figure 6). The resulting vector was then digested with SmaI/PvuII and the oligonucleotide containing fragment was cloned into the pBluescriptKS(+) plasmid (Stratagene, La Jolla, CA), previously digested with PvuII, to yield pUV001 (Figure 6). Several fragments were ligated into pUV001 to create pUV100. The luciferase coding sequences (except first 53 bases) and polyadenylation site were obtained as a 1.8 kilobase XbaI/XmaI fragment from pMLuci (section B-1, Figure 4). The SV40 early splice site and the SV40 late polyadenylation site were obtained as an 871 bp XmaI/BamHI fragment from pMSG (Pharmacia, Piscataway NJ, Figure 7). Both DNA fragments were cloned into pUV001, previously digested with XbaI/BamHI to yield pUV100 (Figure 7).

A 476 b fragment containing a dimeric SV40 polyadenylation site was then cloned into the BclI site of pUV100 (Figure 8). To do this, a 238 bp BclI/BamHI fragment was obtained from SV40 genomic DNA (BRL), ligated, digested with BclI/BamHI, gel isolated, and inserted into pUV100, resulting in the vector pUV100-3 (Figure 8). Linkers containing one SfiI and one NotI restriction site were then cloned into the PvuII/BamHI sites of pUV100-3. Two sets of linkers were synthesized containing the SfiI site in opposite orientations (oligonucleotides D-link1 and D-link2 and oligonucleotides R-link1 and R-link2). The sequences of the oligonucleotides (SEQ ID NO: 7-10) were:

5' GATCGGCCCCTAGGGCCGCGGCCGCAT 3' (D-link1)
5' ATGCGGCCGCGGCCCTAGGGGCC 3' (D-link2)
5' GATCGGCCCTAGGGGCGGCCGCAT 3' (R-link1)
5' ATGCGGCCGCGGCCCCCTAGGGCC 3' (R-link2)

The plasmid that contains D-link oligonucleotides was named pUV102 and the plasmid that contains R-link oligonucleotides was named pUV103 (Figure 9).

The neomycin resistance gene (neo) was then placed under control of the Herpes Simplex Virus thymidine kinase (HSV-TK) promoter to generate a resistance cassette which is free of known enhancer sequences. To do this the HSV-TK promoter was synthesized using four oligonucleotides (Figure 10) (SEQ ID NO: 11-14) designed according to published sequence information (59), and including an SfiI restriction site 5' of the HSV-TK sequences. These oligonucleotides were phosphorylated, annealed, ligated and inserted into pUV100 digested previously with HindIII/NheI, generating the vector pTKL 100 (Figure 11). After verifying the HSV-TK sequence,

the about 3.5 kb NheI/SmaI fragment was isolated from pTKL100, and the about 0.9 kb BstBI/BglII fragment containing the neo coding region was isolated from pRSVNEO (60). These two fragments were filled in with Klenow polymerase and ligated to form pTKNEO (Figure 12). An additional SfiI site was then inserted 3' of the neo gene by isolating the about 1.8 kb SfiI/BamHI and about 2.6 kb SfiI/PVUII fragments of pTKNEO and conducting a three way ligation along with a synthesized SfiI oligonucleotide generating pTKNEO2 (Figure 13) (SEQ. ID NO: 15-16). The HSV-TK/NEO vector containing an optimized Kozac sequence was also utilized (Stratagene, La Jolla, CA, pMC1NEO). An additional vector was constructed by replacing the about 0.9 kb EcoRI/SalI fragment of pTKNEO2 with the about 0.9 kb EcoR1/SalI fragment from pMC1NEO. This vector was termed pTKNEO3. (Figure 14). The SfiI fragment of pTKNEO3, containing the TK promoter and the neomycin resistance gene, was cloned into the SfiI site of pUV102 to yield pUV106 (ATCC # 40946).

C. HGH Reporter Vectors

1. Initial human growth hormone (hGH) promoter-luciferase fusion plasmid

The SalI-XhoI fragment of pSVLuci containing the luciferase coding sequences and the SV40 late polyadenylation site was inserted into pUC 8 (Biorad, Richmond, CA), which had been linearized by a SmaI/HincII digestion and ligated to XhoI linkers (New England Biolabs, Beverly, MA). The new plasmid thus generated (pUXLuci; Figure 15) was linearized by XhoI digestion followed by incubation with the Klenow fragment of *E.*

coli DNA polymerase and the four deoxyribonucleotides to fill in the single-stranded ends of the vector. This linear (5.1 KB) form of pUXLuci was then ligated to the filled-in 550bP HindIII-XbaI fragment of the plasmid phGH:CAT (Figure 16). Human growth hormone promoter sequences located on the HindIII-XbaI fragment were thus fused to the luciferase coding sequences located on pUXLuci generating the plasmid phGH-Luci (Figure 17), which was used in transfections of GC cells as described below. The cell lines resulting from transfections using this vector were used for the high throughput screen as described below.

2. hGH First Intron Construct

Two oligonucleotides, hGH-1 and hGH-4, were used to amplify the human growth hormone region by polymerase chain reaction from human placental genomic DNA. hGH-1 corresponds to bp 4835-4866 (67). hGH-4 corresponds to bp 5557-5586. The PCR reaction yielded a 751 bp DNA fragment comprising the required 5' regulatory elements, the 1st exon. The 1st intron and part of the 2nd exon.

This fragment (called fragment A) was used as a template for a second PCR reaction using oligonucleotides hGH-1 and hGH-3. hGH-3 has the following sequence (SEQ ID NO: 17):

5'-GCC AAA AGC CAT* GG*G CAG GGA CGT CCG GGA-3'

The sequence corresponds to bp 5497-5526 except at the two bases indicated by the *. These two changes will create an in-frame NcoI site in the second exon.

This PCR reaction generated a 691 bp NcoI fragment which

was gel purified and cloned into the NcoI site of puvloz. For both the first-intron and 500 bp constructs, the TK-NEO 3 cassette was inserted into the Sfi I site. The vector linearized and transfected into RAT GC cell to generate growth hormone reporter cell lines.

The resulting plasmid comprises an hGH-luciferase fusion wherein the hGH promoter, first exon, first intron and part of the second exon are fused, in frame. The resulting spliced RNA codes for a chimeric hGH-luciferase fusion protein with luciferase activity.

3. "500 bp" construct:

Fragment A was used as a template for a third PCR reaction using oligonucleotides hGH-1 and hGH-2. hGH-2 has the following sequence (SEQ ID NO: 18):

5' TTA CCT GTA GCC ATG* GCC GCT AGG TGA GCT 3'

The sequence corresponds to bp 5208-5237 except at the bp indicated by the *. This change generates and NcoI site at the hGH initiation codon. This 402 bp fragment was digested with NcoI and inserted into the NcoI site of puvloz. The resulting vector fused the hGH 5' regulatory sequences and 5' untranslated leader to the luciferase open reading frame such that the hGH initiation codon becomes the first codon of luciferase.

4. Quality Control of HGH Reporter Plasmids

The correctness of the above described constructions is confirmed by restriction analysis and DNA sequencing. In addition the plasmids are functionally tested in transient transfections into rat pituitary GC cells for

correct response to known modulators of hGH expression. Electroporations are carried out as described below. 24 hour after transfection cells are treated with 10 - 100 nM rat growth hormone releasing factor or 10 μM forskolin or 1 μM dexamethasone. 4 - 12 hours after treatment cells are lysed by detergent and luciferase activity determined in a scintillation counter as described below.
Additional agents inducing hGH transcription, which could be used for plasmid quality control, include retinoic acid, 12-O-tetradecanoyl-phorbol-13-acetate, 8-bromo-cAMP, Somatostatin, Activin-A, thyroid hormone, and Insulin-like Growth factor I (IGF-I).

D. neu (c-ErbB2) reporter vector

Oligonucleotide probes based on the published sequence (61) of the 5' region of the c-ErbB2 gene were synthesized and used to screen a human leukocyte genomic library (Clontech Inc.). A 3.2 kb BglI fragment from a positive plaque, containing the upstream regulatory elements, the 5' untranslated leader and exon 1 was then subcloned into pBluscriptKS(+), generating pNEU001. A 1.8 kb HincII-NcoI fragment from pNEU001, containing the upstream regulatory elements and most of the 5' untranslated leader was purified by preparative gel electrophoresis and ligated into pUV103 previously digested with SnaBI and NcoI, generating pNEU002. Two oligonucleotides (SEQ ID NO: 19-20) were synthesized:

5'-CATGGGGCCGGAGCCGCAGTGAGCAC-3' and
5'-CATGGTGCTCACTGCGGCTCCGGCCC-3'

These oligonucleotides were annealed to one another, phosphorylated and ligated into NcoI digested pNEU002, generating pNEU103. The synthetic linker fuses the DNA

coding for the neu 5' untranslated leader to the luciferase open reading frame such that the AUG utilized for translation initiation of the neu gene forms the first codon of the luciferase gene. The ScaI-XbaI fragment of pNEU103, containing vector sequences, the upstream regulatory elements, the 5'untranslated leader and a portion of the luciferase open reading frame, was purified by preparative gel electrophoresis and ligated into pUV106 which had previously been digested with ScaI and XbaI, generating pNEU106 (Figure 18). Linearized pNEU106 was used in the transfections to generate the neu-luciferase reporter cell lines as described below.

E. K-ras reporter vector

Oligonucleotides based on the published K-ras sequence (62) were used to isolate two genomic clones by standard methods from a human leukocyte library (Clontech). DNA from these two phages was subcloned into pBluscriptKS(+) (Stratagene) generating pKS4 and pKS11.
A 4 kb XhoI-StuI fragment of pKS11, containing most of intron 1 and exon 1 up to a point 11 bases 5' of the point of translation initiation, was isolated by preparative gel electrophoresis and ligated into XhoI-StuI digested pGEM7Zf (Promega) which had been previously modified by inserting an adaptor the ApaI and XhoI sites in the original vector. This adaptor comprised of two oligonucleotides (5'-TCGAGATCTGAGGCCTGCTGACCATGGGGGCC-3' and
5'-CCCATGGTCAGCAGGCCTCAGATC-3') (SEQ ID NO: 21-22) annealed to one another and was used to allow the proper alignment of the K-ras ATG initiator codon with the luciferase ORF in the final construct (below). The resulting plasmid was designated pGEM715.

A 3 kb HinDIII-XhoI fragment from pKS4, comprising 2.2 kb of K-ras untranscribed upstream DNA and sequences coding for exon 0 and part of intron 1 was purified by preparative gel electrophoresis and ligated into pGEM715 which ad been previously digested with HinDIII and XhoI to generate pGEM7.

A 7.7 kb HinDIII-NcoI fragment of pGEM7, comprising 2.2 kb of K-ras upstream regulatory elements, exon 0, intron 1, and part of exon 1 (to the ATG at the NcoI site), was purified by preparative gel electrophoresis and ligated int pUV102 which had previously been digested with HinDIII and NcoI to generate pKRAS102. The TK-Neo fragment from pTKNeo3 was then ligated into the SfiI site of pKRAS102 to generate pKRAS106 (Figure 19), the vector used for transfections to generated the stable reporter cell lines.

F. CMV reporter vector

A 580 bp cytomegalovirus genomic fragment containing the immediate early promoters and enhancers (63) was ligated into pUV100 previously digested with NotI and NheI and rendered blunt ended by treatment with Klenow fragment, generating pUVCM. An 888 bp NaeI-XbaI fragment from pUVCM, including the CMV promoter and enhancers plus a portion of the luciferase coding region, was purified by preparative gel electrophoresis and ligated into pUV106 which had previously been digested with SnaBI and XbaI, generating pCMV106, the vector used to transfect the CMV reporter cell lines.

G. Transfection

Cell were transfected by one of three methods, following

manufacturer's instructions; by Calcium phosphate precipitation (Pharmacia), Lipofection (Life Technologies Inc.) or electroporation (BioRad). In most cases, 25-75 μg of plasmid DNA, linearized by a single restriction endonuclease cut within the vector sequences, was electroporated into approximately 5 million cells. When co-transfection of a separate neomycin resistant plasmid was employed the molar ratio of luciferase fusion plasmid to neomycin resistant plasmid was either 10:1 or 20:1. Neomycin resistant clones were selected by growth in media containing G418 (Geneticin, Gibco).

H. Liquid Scintillation Counter Bioluminescence Assay

To assay for luciferase expression in transient expression assays in the various transfected clones, cells were incubated with various transcriptional inducers in serum free defined media, washed 3 times with Dulbecco's phosphate-buffered saline (D-PBS, Gibco) and lysed in Lysis Buffer 1 (50 mM Tris acetate pH 7.9, 1 mM EDTA, 10 mM magnesium acetate, 1 mg/ml bovine serum albumin [BSA], 0.5% Brij 58, 2 mM ATP, 100 mM dithiothreitol [DTT]). All reagents were obtained from Sigma except for DTT which was from Boehringer Mannheim. After lysis, cell debris was sedimented by brief centrifugation, and 950 μl of supernatant extract were added to a glass scintillation vial. Samples were counted individually in an LKB (Gaithersburg, MD) scintillation counter on a setting which allows measurement of individual photons by switching off the coincidence circuit. The reaction was started by addition of 50 μl of 2mM luciferin (Sigma, St. Louis, MO or Boehringer Mannheim, Indianapolis IN) in Buffer B (Buffer B-Lysis Buffer 1 without Brij 58, ATP and DTT) to

the 950 μl of lysate. Measurement was started 20 seconds after luciferin addition and continued for 1 minute. Results were normalized to protein concentration using the Bradford protein assay (BioRad, Richmond CA) or to cell numbers using Trypan Blue (Sigma) exclusion counting in a hemocytometer.

I. Construction of Single Cell Clones Containing Various Promoter-Luciferase Fusion Constructs

1. HGH Cell lines

phGH-LUCI and pRSVNeo, an antibiotic resistance plasmid, were co-transfected into GC rat pituitary cells as described above. Selection of G418-resistant cell clones was described above except for using a concentration of 0.2 mg/ml G418. Analysis of the cell clones was performed as above, except that known inducers of hGH expression (10-100 nM rat growth hormone releasing factor (rGRF, Bachem, Torrance, CA) and 10μm forskolin (Sigma, St. Louis, MO) were used in place of dexamethasone. One clone, 532 (ATCC # 10663), was selected for further use in the high throughput screen.

The other HGH reporter plasmids described above are transfected into the rat pituitary cell line GC by electroporation using a BRL (Gaithersburg, Maryland) Cellporator electroporation device. Cells are trypsinized, treated with Soybean trypsin inhibitor (1 mg/ml), washed three times in Dulbecco's modified Eagle's medium (DMEM) without pH indicator, and 1 ml of cell suspension in DMEM electroporated at room temperature and at a cell density of 5 million cells per ml at a voltage of 250 V and a capacitance of 1180 microFarad with the electroporation device set at low resistance. About 15

min after electroporation cells are recovered from the disposable electroporation containers (BRL, Gaithersburg, Maryland) and plated in DMEM and Ham's F12 (1:1) containing 12.5 % fetal calf serum. 2 days after electroporation 0.4 mg/ml G 418 is added and clonal cell colonies picked 14 - 20 days after transfection. Clones are analyzed for correct integration of the promoter-reporter construct, and for correct response to known modulators of hGH expression.

2. c-erbB2 cell line

75 micrograms of the pNEU106 plasmid was linearized by a single restriction endonuclease cleavage within the vector backbone and electroporated into HTB30 human breast carcinoma cells. Neomycin resistant clones were isolated and tested for luciferase activity. Clones testing positive for luciferase production were subjected to Southern blot analysis (see below). The best clone (producing the highest signal and carrying a single intact copy of the transfected DNA) was utilized for high throughput screening (designated clone N-2).

3. MMTV control cell line

pMluci and pSV2Neo, an antibiotic resistance plasmid (64), were co-transfected into NIH/3T3 mouse fibroblast cells using the calcium phosphate precipitation method (65) with a commercially available kit (Pharmacia, Piscataway NJ). Two days later, cells were transferred to media containing 0.4 mg/ml G418 and were grown for an additional 10-14 days. G418-resistant clones were isolated by standard methods. Once sufficient cell numbers were obtained, clones were analyzed based on several criteria: constitutive luciferase production,

induction of luciferase expression by dexamethasone (1 μm, Sigma, St. Louis, MO), satisfactory attachment to microtiter plates used in the high-throughput screen (see section G) and acceptable standard deviation in multiple luciferase expression assays (see below for assay protocol). This analysis was carried out using the luciferase assay conditions described above. Of the clones which satisfied the above criteria for the high throughput screen, one clone, M10 (ATCC # 10659), was selected for use.

4. K-ras control cell line

75 micrograms of the pKRAS106 plasmid was linearized by a single restriction endonuclease cleavage within the vector backbone and electroporated into SW480 human colon carcinoma cells. Neomycin resistant clones were isolated and tested for luciferase activity. Clones testing positive for luciferase production were subjected to Southern blot analysis (see below). The best clone (producing the highest signal and carrying a single intact copy of the transfected DNA) was utilized for high throughput screening (designated clone K-2).

5. CMV control cell line

Hep3B hepatocellular carcinoma cells were transfected by electroporation with 75 micrograms of pCM106 which had been linearized by a single ScaI cut within the vector backbone. Neomycin resistant colonies were isolated and tested for luciferase activity. Luciferase positive, neomycin resistant clones were subjected to Southern blot analysis (see below). The best clone, producing the most luciferase activity from a single, correctly integrated vector was selected for use as the CMV reporter cell line

in the high throughput screen (this clone was designated CM1).

J. High-Throughput (HTP) Screening

Cell plating: Dynatech Microliter 96 well plates were custom pretreated for cell attachment by Dynatech Laboratories, Inc.(Chantilly, VA). Alternatively, the 96 well plates were treated with 50 ul per well of human fibronectin (hFN, 15 μg/ml in PBS, Collaborative Research, Bedford, MA) overnight at 37°C. hFN-treated plates were washed with PBS using an Ultrawash 2 Microplate Washer (Dynatech Labs), to remove excess hFN prior to cell plating. M10 and G21 cells maintained in their respective serum media (with 0.2 mg/ml G418) were washed with PBS, harvested by trypsinization, and counted using a hemocytometer and the Trypan Blue exclusion method according to protocols provided by Sigma, St. Louis, MO Chemical Company. Cells were then diluted into serum free defined media (with 0.2 mg/ml G418), and 0.2 ml of cell suspension per well was plated onto Dynatech treated plates (G21) or hFN-treated plates (M10) using a Cetus Pro/Pette (Cetus, Emeryville CA). Plates were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere.

Addition of Chemicals to Cells: Chemicals from the Oncogene Science file were dissolved in DMSO at concentrations of 3-30 mg/ml. A liquid handling laboratory work station (RSP 5052, Tecan U.S. Chapel Hill, NC) was used to dilute the chemicals (three dilutions; 5 fold, 110 fold, and 726 fold). 10 μl of each dilution were added to each of quadruplicate samples of cells contained in the wells of 96-well Dynatech Microlite Plates. Cell plates were then shaken on a

microplate shaker (Dynatech, medium setting, 30 sec.) and incubated for 6 hours at 37°C, 5% CO_2.

Bioluminescence Assay: After incubation with OSI-file chemicals, cell plates were washed 3 times with PBS using an Ultrawash 2 Microplate Washer (Dynatech Labs) and 75 ul of Lysis Buffer 2 were added to each well (Lysis Buffer 2 is the same as Lysis buffer 1 except that the ATP and DTT concentrations were changed to 2.67 mM and 133 mM, respectively). Bioluminescence was initiated by the addition of 25 ul 0.4 μm Luciferin in Buffer B to each well, and was measured in a Dynatech ML 1000 luminometer following a 1 minute incubation at room temperature. Data were captured using Lotus-Measure (Lotus) software and processed by custom-designed macros written in Lotus.

More recently the cell lysis buffer was modified to also contain the luciferin. Therefore, lysis of cells and the bioluminescence reaction begin simultaneously and the production of bioluminescent light reaches a maximum at about 5 min. The level of light output declines by about 20% within further 30 min. For better lysis buffer stability bovine serum albumin has been omitted. This improved lysis buffer has been shown to remain fully functional for at least 12 hours, when kept on ice and protected from direct light.

Also, more recently, a fully automated device as described in U.S. patent application #382,483 was used to incubate luciferase reporter cells in 96-well microtiter plates, transfer chemicals and known transcriptional modulators to the cells, incubate cells with the chemicals, remove the chemicals by washing with PBS, add lysis buffer to the cells and measure the bioluminescence

produced.

An additional recent improvement is the ability to screen suspension cell lines in the automated high through-put mode using custom manufactured, opaque, 96 well filter plates (Millititer Plates, Millipore Corp.). This involved the manufacture of a robotic filtration and washing station.

RESULTS

A. In vivo signal half-life of the luciferase reporter system

When screening for inhibitors rather than inducers of transcription, the half-life of the reporter molecule becomes a crucial parameter in determining the minimal incubation time that would be necessary to allow enough decay of reporter molecules so that the inhibition of their synthesis became visible. The oncogene reporter cell line were therefore tested for the time dependency of luciferase activity after treatment of the cells with Actinomycin D, an inhibitor of transcription. This experiment measured the combined half-life of luciferase mRNA and of the luciferase protein and compares the rate of signal decay of the H-ras, K-ras and c-erbB2 reporter cell lines to a CMV reporter cell line control. Cells derived from clones CM1 (CMV), K-2 (K-ras) (ATCC # 10662), H21 (H-ras) (ATCC # 10640), plasmid construction details and tranfection information not shown) and N-2 (c-erbB2) (ATCC # 10658) were seeded into 96-well microtiter plates and incubated overnight. At time 0, Actinomycin D (25 μg/ml) was added. At the times indicated in Figure 20, cells were washed with PBS and

luciferase activity of Actinomycin-treated cells determined as described in Materials and Methods. The signal from the treated cells was compared to the luciferase activity of untreated controls. The logarithm of the treated/untreated ratio was plotted versus time, this data is shown in Figure 20. The calculated half-life of the signal from each of the four cell lines is shown in table 1. The half-lives were found to range from about 3 to 10 hours indicating that a 24 hour incubation with a 100% efficient inhibitor of transcription would be sufficient to reduce luciferase levels to 6% of the control in the tested cell lines.

B. Quality Assurance Analysis

A number of quality assurance criteria are routinely assessed during the course of high throughput screens. Data from QA analysis of a portion of Screen III are shown in Figures 21-24. Figure 21 shows an analysis of the consistency of the luciferase signal on various areas of each plate. The ratios of negative control values from three different areas within each plate are calculated and plotted versus plate number. The expected value is 1.0. Values greater than 1.5 or less than 0.4 indicate uneven signal generation across the plate. In this example 240 plates, representing 1440 compounds, tested against three cell lines, are shown. The coefficient of variance for the 12 negative control values from each of the same 240 plates are represented by the data shown in Figure 22. Values less than 20% are considered acceptable. Similar data for the 12 positive control values of the same plates are shown in figure 23. Figure 24 shows the transcription induction ratio (TIR) for the positive controls of one cell line represented in the same set of 240 plates. The TIR is the ratio of the

experimental values to the untreated controls. In this case the cell line is the K-ras reporter and the positive control is Actinomycin D a potent general inhibitor of transcription. Three values are shown for each plot, representing three different concentrations of Actinomycin D. The expected value for such an analysis depends on the half life of the signal and the incubation time (here 24 hours), but for this combination, typical values range from 0.4 to 0.3 fold.

C. High-Throughput Drug Screen

1. Screen I

Table 1 shows a summary of the results of a one-week, high-throughput screen of 2,000 chemicals to identify those chemicals specifically stimulating or inhibiting transcription from the HGH or MMTV and G-CSF (control) promoters. This screen concurrently tested chemicals at three concentrations on quadruplicate samples of the M10 (MMTV), G21 (G-CSF) and 532 (HGH) cell lines. A minimum stimulation of one promoter, to the degree indicated, and less than 50% activation of the other promoter was required for a chemical to be considered a selective activator. A minimum inhibition of 3 fold of one promoter and less than 20% inhibition of the other promoter was required for a chemical to be considered a selective inhibitor. Table 2 identifies the compounds which scored as positive in the screen and reports their induction ratios.

2. Screen II

Table 3 presents the data from another, independent screen representing a three week high throughput screen

of 2334 compounds. Three cell lines were utilized; CM1 (the CMV reporter cell line) as a control for nonspecific effects. N-2 (the c-*erb*B2 reporter cell line) and K-2 (the K-*ras* reporter cell line, also used here as a control). Each compound was assayed at three concentrations in quadruplicate. Each microtiter plate included a negative control row (no added compound) and a positive control row (Actinomycin D at three concentrations). The data are reported as TIR (transcription induction ratio) which is the median of the samples quadruplicate values divided by the median of the negative control values. In this case transcriptional inhibitors are sought, so the selection criteria for lead compounds is that the test promoter be inhibited to 0.4 of the negative control while the other cell lines remain within 0.8X of the control value. During these three weeks 10 compounds scored positive for the specific inhibition of the K-*ras* promoter, 19 scored as leads for the inhibition of the c-*erb*B2 promoter and 39 compounds inhibited nonspecifically.

TABLE 1

SUMMARY OF HIGH-THROUGHPUT SCREEN III

Number (%) of Chemicals Which Activate Expression:

	2-3X	3-5X	5-7X	7-10X	>10X	Total
G-CSF:	0 (0%)	23 (1.1%)	10 (0.5%)	3 (0.15%)	2 (0.10%)	38 (1.9%)
MMTV:	15 (0.7%)	1 (0.05%)	0 (0%)	1 (0.05%)	1 (0.05%)	18 (0.9%)
hGH:	0 (0%)	0 (0%)	12 (0.6%)	5 (0.03%)	6 (0.03%)	23 (1.14%)

Number (%) of Chemicals Which Inhibit Expression >3 Fold

Promoter		
G-CSF	7	(0.35%)
MMTV	1	(0.05%)
hGH	42	(2.1 %)

TABLE 2

A) SCREEN III TRANSCRIPTIONAL ACTIVATORS

Chemical#	Chemical Name	FOLD INDUCTION RELATIVE TO SOLVENT CONTROL		
		GCSF	hGH	MMTV
G-CSF:				
40	3-Acetyl-2-6-Bis(tertiary butyl amino)-4-methyl-pyridine	5.62	0.62	0.27
58	1-Acetylimidazole	6.03	0.17	0.42
237	N-Carbethoxy-phthalimide	4.77	0.06	0.62
254	1-(2-Chloroethyl)piperidine	4.09	0.90	0.98
364	Melamine	3.67	1.18	1.07
473	1,3,5,-Triazine	>3	0.50	0.87
542	5-Bromo-2'-deoxycytidine	6.28	1.08	1.26
543	5-Bromo-2'-deoxyuridine	7.17	0.72	0.98
878	Blueberry leaf extract	3.84	1.17	0.78
1025	Culvers Root extract	4.09	0.98	1.24
1234	4-Aminocinnamic Acid hydrochloride	4.97	0.51	1.03
1255	1-Bromo-3,5-dichlorobenzene	6.74	0.43	1.09
1374	4'-Amino-N-methylacetanilide	11.03	0.05	1.05
1375	4'-(aminomethyl)benzene sulfonamide hydrochloride	8.94	0.04	1.37
1376	2-Amino-5-Methyl benzene sulfonic acid	6.37	0.04	1.32
1397	5-Amino-3-methylisothiazole hydrochloride	3.63	0.57	1.13
1482	2-Aminophenyl disulfide	3.99	0.54	1.07
1483	4-Aminophenyl disulfide	4.64	0.38	1.09

Table 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1521	2-Amino-6-purinethiol	3.59	0.73	0.92
1583	8-Bromoadenosine	5.82	0.12	0.88
1592	Bis(2,2,3,3,4,4,5,5,6,6,7,7,) dodecafluoroheptyl-(+)-camphorate	3.20	0.74	1.34
1783	Cupferron	6.55	0.32	0.89
1793	Cyanomethyl-N,N-dimethyl dithiocarbamate	9.50	0.52	1.21
1994	3-Bromobiphenyl	3.29	0.34	0.63
2001	1-Bromo-4-tertiary butyl benzene	3.11	0.74	1.12
2030	4-Bromo-2-fluoro-6-nitroanizol	5.53	0.67	0.87
2096	(+)-1-Bromo-3-Chloro-2methyl propane	3.27	0.61	0.89
2097	1-Bromo-5-Chloro pentane	5.09	0.88	1.22
2129	4-Chlorobenzyl Chloride	3.23	0.75	0.95
GROUP A:				
378	7-Oxo-7H-benzo[e]pyrimidine 4-carboxylic acid	4.12	0.26	0.59
423	Quinacrine dihydrochloride hydrate	2.39	0.56	0.64
427	Resazurin	3.14	0.43	0.71
836	Thionin	3.20	0.23	0.58
1776	Cresyl Violet Acetate	3.50	0.15	1.36
1904	9-Aminoacridine hydrochloride	4.12	0.54	0.82
GROUP B:				
670	Methyl Green	>3	0.52	0.79
1780	Crystal Violet	20.39	0.38	1.15

Table 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
GROUP A AND B:				
80	Acridine Orange	5.87	0.66	0.83
hGH:				
70	2-Acetylpyrrole	0.43	9.26	0.85
299	10,11-Dihydrocarbamazepine	0.53	5.46	0.47
322	1-ethyl-2-benzimidazolinone	0.60	11.18	1.12
325	Fisetin	0.14	5.42	1.0
552	3-(4-chlorophenyl)-1-methoxy-1-methyl urea	0.81	5.31	0.86
790	Rivanol	0.01	5.94	0.58
792	Rose Bengal	0.94	5.31	1.21
856	Tripaμmitin	0.28	6.49	0.42
1004	Arnica 4x	0.85	6.48	1.22
1160	Rochester # 6180	0.38	5.79	0.80
1251	Bromocresol Green	0.14	15.19	0.33
1337	4-Amino-5-hydroxy-1-naphthalene sulfonic acid	0.07	15.87	0.23
1499	2-Amino-4-phenylthiazole hydrobromide monohydrate	0.24	5.55	0.61
1550	2-Aminothiazole	0.04	5.44	0.87
1552	2-amino-2-thiazoline	1.23	7.26	0.52
1561	4-Amino-3,5,6-trichloropicolinic acid	0.23	8.05	0.48
1598	N,N'-Bis-[3-(4,5-dihydro-1H-imidizol-2-yl)phenyl] urea dipropanoate	0.72	5.32	1.27

Table 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1678	4,8-Bis(hydroxymethyl)-tricyclo [5,2,1,$0^{2.6}$]decane	0.36	7.08	0.89
1740	5-carbethoxy-2-thiouracil	0.74	17.77	0.87
1747	N_6-carbobenzyloxy-L-lysine	0.78	6.16	0.86
1804	Cyclobutane carboxylic acid	1.05	9.41	0.49
1876	Alec Blue	0.87	11.91	0.40
1881	Alizarin Blue Black B	0.21	18.87	0.69
MMTV:				
189	Bathocuproinedisulfonic Acid disodium salt hydrate	1.06	1.47	2.80
453	2,2':6',2"-Terpyridine	0.79	0.58	13.30
519	b-Apo-8'-carotenal	1.15	0.68	2.76
562	Copaiva Balsam	1.10	0.15	2.34
629	Homoveratric acid	0.85	1.05	2.48
633	5-Iodorotic acid	1.02	0.86	2.46
765	Prednisolone-21-Acetate	0.96	1.30	2.66
828	2,4,5,4'-Tetrachlorodiphenylsulfide	1.47	1.34	2.20
848	Triamcinolone acetonide	0.75	1.28	2.43
944	Peanut	1.15	0.91	2.10
1269	5-Amino-4,6-dichloropyrimidine	0.72	0.91	2.18
1316	2-Aminofluorene	0.74	1.39	2.33
1318	2-Amino-9-fluorenone	1.13	0.85	2.41
1384	2-Amino-4'-methylbenzophenone	1.33	0.50	2.43

Table 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1573	5-Bromoacenapthene	1.49	0.34	4.30
2064	4-(Bromomethyl)-6,7-dimethoxy-coumarin	0.82	1.10	2.53
2148	2-chlorocyclohexanone	0.45	0.92	2.82
2191	Chloramphenicol	0.37	0.35	7.32

B) SCREEN III TRANSCRIPTIONAL INHIBITORS

	CONTROL	FOLD INHIBITION RELATIVE TO SOLVENT		
Chemical#	Chemical Name	GCSF	hGH	MMTV
G-CSF:				
209	4-Benzoylpyridine	6.66	1.08	0.81
371	Morin hydrate	11.11	0.41	0.89
660	Maclurin	10.0	0.34	1.04
798	Salicylamide	4.76	0.90	0.68
2009	4-Bromo-3,5-dimethylpyrazole	3.70	0.57	0.64
2082	4-Bromo-3-Methylpyrazole	5.26	0.65	1.23
2121	3-Chlorobenzyl alcohol	4.76	0.40	1.14
hGH:				
183	Auramine O	0.72	4.00	0.70
240	Carminic acid	0.63	5.26	0.80
443	Sulfamethazine	0.60	4.76	0.79
512	Amaranth	0.81	5.26	0.68

Table 2 (CONT.)

Chemical#	Chemical Name	FOLD INHIBITION GCSF	hGH	MMTV
541	5-Bromo-4-Chloro-3-indoxyl-phosphate K-salt	0.90	6.25	0.86
556	Chromazurol S	0.73	33.33	0.87
561	Clove Oil	0.62	5.00	0.05
577	Na-Ne-Diacetyl-L-lysine	0.64	4.00	0.68
578	Dibenzoyl-D-tartaric acid	0.65	4.00	0.91
630	Hydantoin-5-acetic acid	0.70	3.57	0.74
640	Kernechtrot	0.64	5.00	0.59
759	Piperidine	0.64	5.88	0.95
764	Prednisolone	0.82	4.54	0.59
875	Black Walnut extract	0.69	6.25	0.80
892	Colts Foot Leaves extract	0.68	11.11	0.87
893	Comfrey Leaf extract	0.74	11.11	0.90
920	Horehound Herb extract	0.56	3.84	0.84
921	Horsetail Grass extract	0.72	3.44	0.86
942	Pau D'Arco extract	0.80	6.25	0.63
970	Thyme extract	0.57	4.34	1.07
1591	1,2-Bis(di-p-tolylphosphino)-ethane	0.56	5.55	0.96
1604	2,4-Bis[5,6-bis(4-sulfophenyl)-1,2,4-Triazine-3-yl)-pyridine, tetrasodium salt hydrate	0.77	5.00	0.97
1635	[(15)-endo]-(-)-Borneol	0.71	9.09	0.99
1640	1,2-Bis(2-pyridyl)-ethylene	0.79	5.00	0.59
1641	2,3-Bis(2-pyridyl)-pyrazine	0.83	5.55	0.60

Table 2 (CONT.)

Chemical#	Chemical Name	FOLD INHIBITION GCSF	hGH	MMTV
1648	2-[5,6-Bis(4-sulfophenyl)-1,2,4-triazine-3-yl]-4-(4-sulfophenyl)-pyridine, trisodium salt	0.86	7.69	1.00
1651	Bis(2,2,2-trifluoroethyl) (methocarbonyl-methyl)-phosphonate	0.69	3.57	0.70
1655	2,5-Bis(trifluoro-methyl)benzoic acid	0.54	4.76	0.81
1703	3-Bromobenzonitrile	0.76	10.00	0.90
1704	4-Bromobenzonitrile	0.77	4.16	0.94
1705	4-Bromobenzophenone	0.54	14.28	0.62
1712	Calcein Blue	0.74	8.33	0.94
1720	(15)-(-)-Camphor	0.65	4.76	0.66
1764	7-(Carboxymethoxy)-4-Methylcoumarin	0.55	7.14	0.82
1770	Carminic acid	0.54	10.00	0.57
1771	L-Carnosine	0.71	10.00	0.72
1773	O-Cresolphthalein Complexone	0.62	10.00	0.67
1890	Alloxazine	0.80	5.26	0.58
2035	5-Bromofuroic acid	0.57	7.14	0.89
2036	8-Bromoguanosine	0.58	4.34	0.81
2037	1-Bromohexadecane	0.51	4.00	0.50
MMTV:				
2010	2-Bromo-4,6-dinitroaniline	0.80	0.63	3.57

TABLE 3

The Number (and %) of Compounds Scoring as Specific Repressors

Cell Line	Number	%
K-ras	10	0.4
neu	19	0.8
CMV	1	0.04
Toxic	39	1.7

REFERENCES

1. James, R. and Bradshaw, R.A., (1984), Ann. Rev. Biochem. 53:259.
2. Ullrich, A. and Schlessinger, J., (1990), Cell 61:203.
3. Sporn, M.B. and Roberts, A.B., (1988), Nature 332:217.
4. Carpenter, G. and Cohen, S., (1979), Ann. Rev. Biochem. 48:193.
5. Panaretto, B.A., et al., (1984), J. Endocrin. 100:259.
6. Kamata, N. et al., (1986), Cancer Res. 46:1648.
7. Gregory, H., (1975), Nature 257:325.
8. Assoian, R. et al., (1983), J. Biol. Chem. 258:7155.
9. Seyedin, S.M. et al., (1986), J. Biol. Chem. 261:5693.
10. ten Dijke, P. et al., (1988), Proc. Natl. Acad. Sci. USA 85:4715.
11. Jakowlew, S.B., (1988), Mol. Endocrin. 2:1186.
12. Moses, et al., (1981), Cancer Res. 41:2842.
13. Shipley, G.D. et al., (1985), Proc. Natl. Acad. Sci. USA 82:4147.
14. Postlewaste, A.E., (1987), J. Exp. Med. 165:251.
15. Wahl, S.M. et al., (1987), Proc. Natl. Acad. Sci. USA 84:5788.
16. Ignotz, R.A. et al., (1987), J. Biol. Chem. 262:6443.
17. Raghaw, R. et al., (1987), J. Clin. Invest 79:1285.
18. Tucker, R.F. et al., (1984), Science 226:705.
19. Moses, H.L. et al., (1985), Cancer Cells 3:65.
20. Moses, H.L. and Leof, E.B., (1986), In: Oncogenes and Growth Factors, Kahn, P. and Graf, T., eds, pg. 51
21. Nicola, A. and Vador, M., (1987), In: Oncogenes and Growth Factors, Bradshaw, R.A. and Prenter, S., eds, pg. 164
22. Jakobovits, A., (1986), In: Oncogenes and Growth Control, Kahn, P. and Graf, T. eds, pg 9.
23. Morikawa, M. et al., (1982), Cell 29:783.
24. Coussins, L. et al., (1985), Science 230:1132.
25. Shih, C. et al., (1981), Nature 290:261.
26. Slamon, D.J. et al., (1987), Science 235:177.

27. Slamon, D.J. et al., (1989), Science 244:707.

28. Lupu, R. et al., (1990), Science 249:1552.

29. Qiu, F. et al., (1988), EMBO J. 7:1003.

30. Yarden, Y. et al., (1987), EMBO J. 6:3341.

31. Zsebo, K.M. et al., (1990), Cell 63:213.

32. Huang, E. et al., (1990), Cell 63:225.

33. Maniatis, T. et al., (1987), Science, 236:1237.

34. Yanofsky, C. and Crawford, I.P., (1987), in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (F.C. Neidhardt et al., eds.) Vol. 2, p. 1453.

35. Schlief, R. (1987), in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (F.C. Neidhardt et al., eds.) Vol. 2, p. 1473.

36. McClure (1985), Ann. Rev. Biochem., 54:171.

37. Hoopes, B.C. and McClure, W.R. (1987), in Escherichia coli and Salmonella Typhimurium: Cellular and Molecular Biology (F.C. Neidhardt et al., eds.) Vol. 2, p. 1231.

38. Matthews, B.W. (1987), Cro repressor structure and its interaction with DNA. In DNA: Protein Interactions and Gene Regulation (E.B. Thompson and J. Papaconstantinou, eds.) University of Texas Press, Austin.

39. Schlief, R. (1988), Science, 241:1182.

40. Evans, R.M. and Hollenberg, S.M. (1988), Cell, 52:1.

41. Landschulz, W.H., et al., (1988), Science, 240:1759.

42. Levine, M. and Hoey, T. (1988), Cell, 55:537.

43. Krainer, A.R. and Maniatis, T. (1988) RNA splicing. In Transcription and Splicing (Hames, B.D. and Glover, D.M., eds.) IRL Press, Washington, D.C., Vol. 1.

44. Proudfoot, N.J. and Whitelaw, E. (1988), Termination and 3' end processing of eucaryotic RNA. In Transcription and Splicing (Hames, B.D. and Glover, D.M., eds.) ERL Press, Washington, D.C., Vol. 1, p. 97.

45. La Thangue, N.B. and Rigby, P.W.J. (1988), Transacting protein factors and the regulation of eukaryotic transcription. In Transcription and Splicing (Hames, B.D. and Glover, D.M., eds)

IRL Press, Washington, D.C., Vol. 1.

46. Yamamoto, K.R. (1985), Ann. Rev., Genet., 19:209.
47. Denison, M.S., et al., (1988), Proc. Natl. Acad. Sci. USA, 85:2528.
48. Hoeffler, J.P., et al., (1988), Science, 242:1430.
49. Angel, P., et al., (1987), Mol. Cell. Biol., 7:2256.
50. Angel, P., et al., (1987), Cell, 49:729.
51. Edelman, A.M., et al., (1987) Protein Serine/Threonine Kinases Ann. Rev. 56:567-613.
52. Yamamoto, K.K., et al., (1988), Nature, 334:494.
53. De Wet, J.R., et al., (1985), Proc. Natl. Acad. Sci. USA, 82:7870.
54. Engebrecht, J.M., et al., (1985), Science, 227:1345.
55. Bottenstein, J.,et al., (1979), Methods in Enzymology, 58:94.
56. Hayashi, I., et al., (1978), In Vitro, 14:23.
57. Maniatis, T., et al. (1982), Molecular Cloning: A Laboratory Manual Cold Spring Harbor Labs C.S.H. New York.
58. Ow, D.W., et al., (1986), Science 234:856-859.
59. McKnight, S.L. (1982), Cell, 31:355.
60. Gorman, C. (1985) Vectors used in mammalian cell expression. In DNA Cloning, Vol. II (D.M. Glover, ed). IRL Press, Washington, D.C.
61. Hudson, L.G., et al., (1990), J. Biol. Chem. 265:4389-4393.
62. Jordando, J. and Perucho, M. (1988) Oncogene 2: 359-366.
63. Liu, B.,et al., (1991), J. Virol. 65:897-90.
64. Pouwels, Ph.H., Enger-Valk, B.E., and Brammar, W.J. (1985) Cloning Vectors. Elsevier Science Publishers, B.V., Amsterdam.
65. Graham, F.L. and Van der Ed, A.J. (1973), Virology, 52:456.
66. Mizel, S.B. (1989) FASEB J. 3:2379.
67. Chen et al. (1989) Genomics 4:479-497.
68. Edwards, D.R. and Heath, J.K., (1991), Regulation of transcription by transforming growth factor-ß in Hormonal Control of Gene Transcription. (Cohen, P. and Foulkes, G.J., eds.), Elsevier Science Publishers, B.V., Amsterdam, p 333.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Oncogene, Science Inc.

(ii) TITLE OF INVENTION: Methods of Transcriptionally Modulating Expression of Growth Factor Genes and Growth Factor Receptor Genes

(iii) NUMBER OF SEQUENCES: 22

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: John P. White, Esq.
(B) STREET: 30 Rockefeller Plaza
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10112

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 17-JAN-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: White, John P.
(B) REGISTRATION NUMBER: 28,678
(C) REFERENCE/DOCKET NUMBER: 26134-F1-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-977-9550
(B) TELEFAX: 212-664-0525
(C) TELEX: 422523 coop ui

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAACAGTGGT GTGGCGACTC CGTTTAGCTG TTCTGGAGCT
40

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGATCGCAG CGCTGCCTTT CCT
23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATGAGGAAA GGCAGCGCTG CGATCCAGCA C
31

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGGCGCAGCG CTCCAGGAGA AGCTG
25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCTATGGAG TTGGCTCAAG CAGCCTGC
28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGCGGGTCTG TAGGCAGGTC GGCTC
25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGTAAGAGC TCAGCCCTTG CCCTGGGCAG G
31

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCCAGCCCG CAGCTCCAGG AGTCTG
26

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCTCTACAC TGGCAGTTCC ACCTG
25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGCCAAGGAG GCCGAGAATA TCACG

25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCAGACTTC TACGGCCTGC TGCCCGAC
28

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCAGCAATTG AGAGCATTCT TAAA
24

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCCTTGATA TGGATTGGAT GTCG
24

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACTAATAATG TAAAAGACGT CACTAAATTG
30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTCGCTTAT CCAACAATGA CTTGG
25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCAGAACAGC TAAACGGAGT CGCCACACCA CTGTTTGTGC
40

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCCAAAAGCC ATGGGCAGGG ACGTCCGGGA
30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTACCTGTAG CCATGGCCGC TAGGTGAGCT
30

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CATGGGGCCG GAGCCGCAGT GAGCAC
26

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CATGGTGCTC ACTGCGGCTC CGGCCC
26

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCGAGATCTG AGGCCTGCTG ACCATGGGGG CC
32

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCCATGGTCA GCAGGCCTCA GATC
24

What is claimed is:

1. A method of directly transcriptionally modulating the expression of a gene encoding a growth factor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism, which comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the growth factor encoded by the gene which is expressed by the cell, which molecule (a) does not naturally occur in the cell and (b) specifically transcriptionally modulates expression of the gene encoding the growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

2. A method of directly transcriptionally modulating the expression of a gene encoding a growth factor receptor, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism, which comprises contacting a cell, which is capable of expressing the gene, with a molecule at a concentration effective to transcriptionally modulate expression of the gene and thereby affect the level of the growth factor receptor encoded by the gene which is expressed by the cell, which molecule (a) does not naturally occur in the cell and (b) specifically transcriptionally modulates expression of the gene encoding the growth factor receptor, and (c) binds to DNA or RNA, or binds to a

protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

3. A method of claim 1 or 2, wherein the molecule does not naturally occur in any cell of a lower eucaryotic organism.

4. A method of claim 1 or 2, wherein the molecule does not naturally occur in any cell.

5. A method of claim 1 or 2, wherein the molecule is not a naturally occurring molecule.

6. A method of claim 1 or 2, wherein the cell is a cell of the multicellular organism.

7. A method of claim 1 or 2, wherein the cell is a fish cell.

8. A method of claim 1 or 2, wherein the cell is a avian cell.

9. A method of claim 1 or 2, wherein the cell is an animal cell.

10. A method of claim 9, wherein the animal cell is a human cell.

11. A method of claim 9, wherein the animal cell is a bovine cell.

12. A method of claim 9, wherein the animal cell is a

porcine cell.

13. A method of claim 1, wherein the transcriptional modulation comprises upregulation of expression of the gene encoding the growth factor.

14. A method of claim 1, wherein the transcriptional modulation comprises downregulation of expression of the gene encoding the growth factor.

15. A method of claim 2, wherein the transcriptional modulation comprises upregulation of expression of the gene encoding the growth factor receptor.

16. A method of claim 2, wherein the transcriptional modulation comprises downregulation of expression of the gene encoding the growth factor receptor.

17. A method of claim 1 or 2, wherein the molecule binds to a modulatable transcriptional sequence of the gene.

18. A method of claim 1 or 2, wherein the molecule comprises an antisense nucleic acid.

19. A method of claims 17, wherein the molecule comprises double-stranded nucleic acid.

20. A method of claim 17, wherein the molecule comprises a nucleic acid capable of forming a triple helix with double-stranded DNA.

21. A method of claim 1, wherein the growth factor is a human growth factor.

22. A method of claim 1, wherein the growth factor is a

bovine growth factor.

23. A method of claim 1, wherein the growth factor is a porcine growth factor.

24. A method of claim 1, wherein the growth factor is a fish growth factor.

25. A method of claim 1, wherein the growth factor is an avian growth factor.

26. A method of claim 1, wherein the growth factor is a transforming growth factor beta.

27. A method of claim 1, wherein the growth factor is an epidermal growth factor.

28. A method of claim 1, wherein the growth factor is a transforming growth factor alpha.

29. A method of claim 1, wherein the growth factor is insulin.

30. A method of claim 1, wherein the growth factor is an insulin-like growth factor.

31. A method of claim 1, wherein the growth factor is a platelet derived growth factor.

32. A method of claim 1, wherein the growth factor is a vascular endothelial cell growth factor.

33. A method of claim 1, wherein the growth factor is a fibroblast growth factor.

34. A method of claim 1, wherein the growth factor is a nerve growth factor.

35. A method of claim 1, wherein the growth factor is a an interleukin.

36. A method of claim 35, wherein the interleukin is IL-1.

37. A method of claim 35, wherein the interleukin is IL-2.

39. A method of claim 35, wherein the interleukin is IL-4.

40. A method of claim 35, wherein the interleukin is IL-5.

41. A method of claim 35, wherein the interleukin is IL-6.

42. A method of claim 1, wherein the growth factor is a bone morphogenic protein.

43. A method of claim 2, wherein the growth factor receptor is a human growth factor receptor.

44. A method of claim 2, wherein the growth factor receptor is a bovine growth factor receptor.

45. A method of claim 2, wherein the growth factor receptor is a porcine growth factor receptor.

46. A method of claim 2, wherein the growth factor receptor is a fish growth factor receptor.

47. A method of claim 2, wherein the growth factor receptor is an avian growth factor receptor.

48. A method of claim 2, wherein the growth factor

receptor is the receptor for a transforming growth factor beta.

49. A method of claim 2, wherein the growth factor receptor is the receptor for an epidermal growth factor.

50. A method of claim 2, wherein the growth factor receptor is the receptor for a transforming growth factor alpha.

51. A method of claim 2, wherein the growth factor receptor the receptor for insulin.

52. A method of claim 2, wherein the growth factor receptor is a platelet derived growth factor receptor.

53. A method of claim 2, wherein the growth factor receptor is VEGF receptor.

54. A method of claim 2, wherein the growth factor receptor is NGF receptor.

55. A method of claim 2, wherein the growth factor receptor is an interleukin receptor.

56. A method of claim 55, wherein the interleukin receptor is an IL-1 receptor.

57. A method of claim 55, wherein the interleukin receptor is IL-2 receptor.

58. A method of claim 55, wherein the interleukin receptor is IL-2α receptor.

59. A method of claim 55, wherein the interleukin receptor is IL-3 receptor.

60. A method of claim 2, wherein the growth factor receptor is a hematopoietic growth factor receptor.

61. A method of claim 60, wherein the hematopoietic growth factor receptor is a G-CSF receptor.

62. A method of claim 60, wherein the hematopoietic growth factor receptor is GM-CSF receptor.

63. A method of claim 60, wherein the hematopoietic growth factor receptor is EPO receptor.

64. A method of claim 60, wherein the hematopoietic growth factor receptor is c-<u>fms</u> (M-CSF receptor).

65. A method of claim 60, wherein the hematopoietic growth factor receptor is c-<u>kit</u> (c-<u>kit</u> ligand receptor).

66. A method of claim 2, wherein the growth factor receptor is <u>erb</u>B2 (<u>neu</u>).

67. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the gene encoding the growth factor, and (iii) a

DNA sequence encoding a polypeptide other than the growth factor, which polypeptide is capable of producing a detectable signal, which DNA sequence is coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable detectable signal to be produced by the polypeptide so expressed, quantitatively determining the amount of the signal produced, comparing the amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

68. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the gene encoding the growth factor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene

encoding the growth factor, causes a measurable change in the amount of the polypeptide produced, quantitatively determining the amount of the polypeptide so produced, comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the amount of the polypeptide expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

69. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor, which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor, (ii) a promoter of the gene encoding the growth factor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence, quantitatively determining the amount of the mRNA produced, comparing the amount so determined with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule

as one which causes a change in the detectable mRNA amount of, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor.

70. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of the gene encoding the growth factor receptor, and (iii) a DNA sequence encoding a polypeptide other than the growth factor receptor, which polypeptide is capable of producing a detectable signal, which DNA sequence is coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor receptor, causes a measurable detectable signal to be produced by the polypeptide so expressed, quantitatively determining the amount of the signal produced, comparing the amount so determined with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable signal produced by the polypeptide so expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor

receptor.

71. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of the gene encoding the growth factor receptor, and (iii) a reporter gene, which expresses a polypeptide, coupled to, and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor receptor, causes a measurable change in the amount of the polypeptide produced, quantitatively determining the amount of the polypeptide so produced, comparing the amount so determined with the amount of polypeptide produced in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the amount of the polypeptide expressed, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

72. A method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene encoding a growth factor receptor, which comprises contacting a sample

which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene encoding the growth factor receptor, (ii) a promoter of the gene encoding the growth factor receptor, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene encoding the growth factor receptor, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence, quantitatively determining the amount of the mRNA produced, comparing the amount so determined with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the detectable mRNA amount of, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene encoding the growth factor receptor.

73. A method of claim 67, 68, 69, 70, 71 or 72, wherein the sample comprises cells in monolayers.

74. A method of claim 67, 68, 69, 70, 71 or 72, wherein the sample comprises cells in suspension.

75. A method of claim 67, 68, 69, 70, 71 or 72, wherein the cells comprise animal cells.

76. A method of claim 75, where the animal cells are human cells.

77. A method of claim 75, where the animal cells are bovine cells.

78. A method of claim 75, where the animal cells are murine cells.

79. A method of claim 75, where the animal cells are porcine cells.

80. A method of claim 75, where the animal cells are fish cells.

81. A method of claim 75, where the animal cells are avian cells.

82. A method of claim 67, 68, 69, 70, 71 or 72, wherein the predefined number of cells is from about 1 to about 5 X 10^5 cells.

83. A method of claim 82, wherein the predefined number of cells is from about 2 X 10^2 to about 5 X 10^4 cells.

84. A method of claim 67, 68, 69, 70, 71 or 72, wherein the predetermined amount of the molecule to be tested is based upon the volume of the sample.

85. A method of claim 67, 68, 69, 70, 71 or 72, wherein the predetermined amount is from about 1.0 pM to about 20 μM.

86. A method of claim 67, 68, 69, 70, 71 or 72, wherein the predetermined amount is from about 10 nM to about 500 μM.

87. A method of claim 67, 68, 69, 70, 71 or 72, wherein

the contacting is effected from about 1 to about 24 hours.

88. A method of claim 87, wherein the contacting is effected from about 2 to about 12 hours.

89. A method of claim 67, 68, 69, 70, 71 or 72, wherein the contacting is effected with more than one predetermined amount of the molecule to be tested.

90. A method of claim 67, 68, 69, 70, 71 or 72, wherein the molecule to be tested is a purified molecule.

91. A method of claim 67, 68, 69, 70, 71 or 72, wherein the modulatable transcriptional regulatory sequence comprises a cloned genomic regulatory sequence.

92. A method of claim 67, 68, 69, 70, 71 or 72, wherein the DNA consists essentially of more than one modulatable transcriptional regulatory sequence.

93. A method of claim 67, 68, 70 or 71, wherein the DNA sequence encoding the polypeptide is inserted downstream of the promoter of the gene encoding a growth factor by homologous recombination.

94. A method of claim 67 or 70, wherein the polypeptide is a luciferase.

95. A method of claim 67 or 70, wherein the polypeptide is chloramphenicol acetyltransferase.

96. A method of claim 67 or 70, wherein the polypeptide is β glucuronidase.

97. A method of claim 67 or 70, wherein the polypeptide is β galactosidase.

98. A method of claim 67 or 70, wherein the polypeptide is neomycin phosphotransferase.

99. A method of claim 67 or 70, wherein the polypeptide is guanine xanthine phosphoribosyltransferase.

100. A method of claim 67 or 70, wherein the polypeptide is alkaline phosphatase.

101. A method of claim 68 or 71, wherein the polypeptide is capable of complexing with an antibody.

102. A method of claim 68 or 71, wherein the polypeptide is capable of complexing with biotin.

103. A method of claim 69 or 72, wherein mRNA is detected by quantitative polymerase chain reaction.

104. A screening method according to any of claims 67, 68, 69, 70, 71 or 72 which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested.

105. A screening method of claim 104, wherein the plurality of samples comprises more that about 10^4 samples.

106. A screening method of claim 104, wherein the plurality of samples comprises more than about 5×10^4 samples.

107. A method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding growth factors which comprises essentially simultaneously screening the molecules against the genes encoding the growth factors according to the method of claim 104.

108. A method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes encoding growth factor receptor(s) which comprises essentially simultaneously screening the molecules against the genes encoding the growth factor receptor(s) according to the method of claim 104.

109. A screening method of any of claims 107 or 108, where more than about 10^3 samples per week are contacted with different molecules.

110. A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding a growth factor, the expression of which is associated with a defined physiological or pathological effect in the organism, which comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect, which molecule (a) does not naturally occur in the organism and (b) specifically transcriptionally modulates expression of the gene encoding a growth factor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the

binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

111. A method for directly transcriptionally modulating in a multicellular organism the expression of a gene encoding a growth factor receptor, the expression of which is associated with a defined physiological or pathological effect in the organism, which comprises administering to the organism a molecule at a concentration effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect, which molecule (a) does not naturally occur in the organism and (b) specifically transcriptionally modulates expression of the gene encoding a growth factor receptor, and (c) binds to DNA or RNA, or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.

112. A method of claim 110 or 111, wherein the molecule comprises an antisense nucleic acid.

113. A method of claim 110 or 111, wherein the molecule comprises a double stranded nucleic acid molecule.

114. A method of claim 110 or 111, wherein the molecule comprises a nucleic acid capable of forming a triple helix with double stranded DNA.

115. A method of claim 110 or 111, wherein the multicellular organism is a human being.

116. A method of claim 110 or 111, wherein the multicellular organism is an animal.

117. A method of claim 116 wherein the animal is a cow.

118. A method of claim 116 wherein the animal is a pig.

119. A method of claim 116 wherein the animal is a fish.

120. A method of claim 116 wherein the animal is a chicken.

121. A method of claim 116 wherein the animal is a mouse.

122. A method of claim 115, wherein the defined pathological effect is a disorder and modulated expression of the gene encoding a growth factor is associated with amelioration of the disorder.

123. A method of claim 115, wherein the defined pathological effect is pituitary dwarfism.

124. A method of claim 115, wherein the defined pathological effect is a acute catabolic trauma.

125. A method of claim 115, wherein the defined pathological effect is obesity.

126. A method of claim 115, wherein the defined pathological effects are the combined degenerative disorders of old age.

127. A method of claim 115, wherein the defined pathological effect is cancer.

128. A method of claim 115, wherein the defined

pathological effect is bladder cancer.

129. A method of claim 115, wherein the defined pathological effect is brain cancer.

130. A method of claim 115, wherein the defined pathological effect is breast cancer.

131. A method of claim 115, wherein the defined pathological effect is lung cancer.

132. A method of claim 115, wherein the defined pathological effect is ovarian cancer.

133. A method of claim 115, wherein the defined pathological effect is colon cancer.

134. A method of any of claims 110 or 111 wherein the administering comprises topical contact.

135. A method of any of claims 110 or 111, wherein the administering comprises oral, transdermal, intravenous, intramuscular or subcutaneous administration.

Figure 1.
Features of the Mammalian Vector pUV102 with Inserted TK-NEO Cassette.

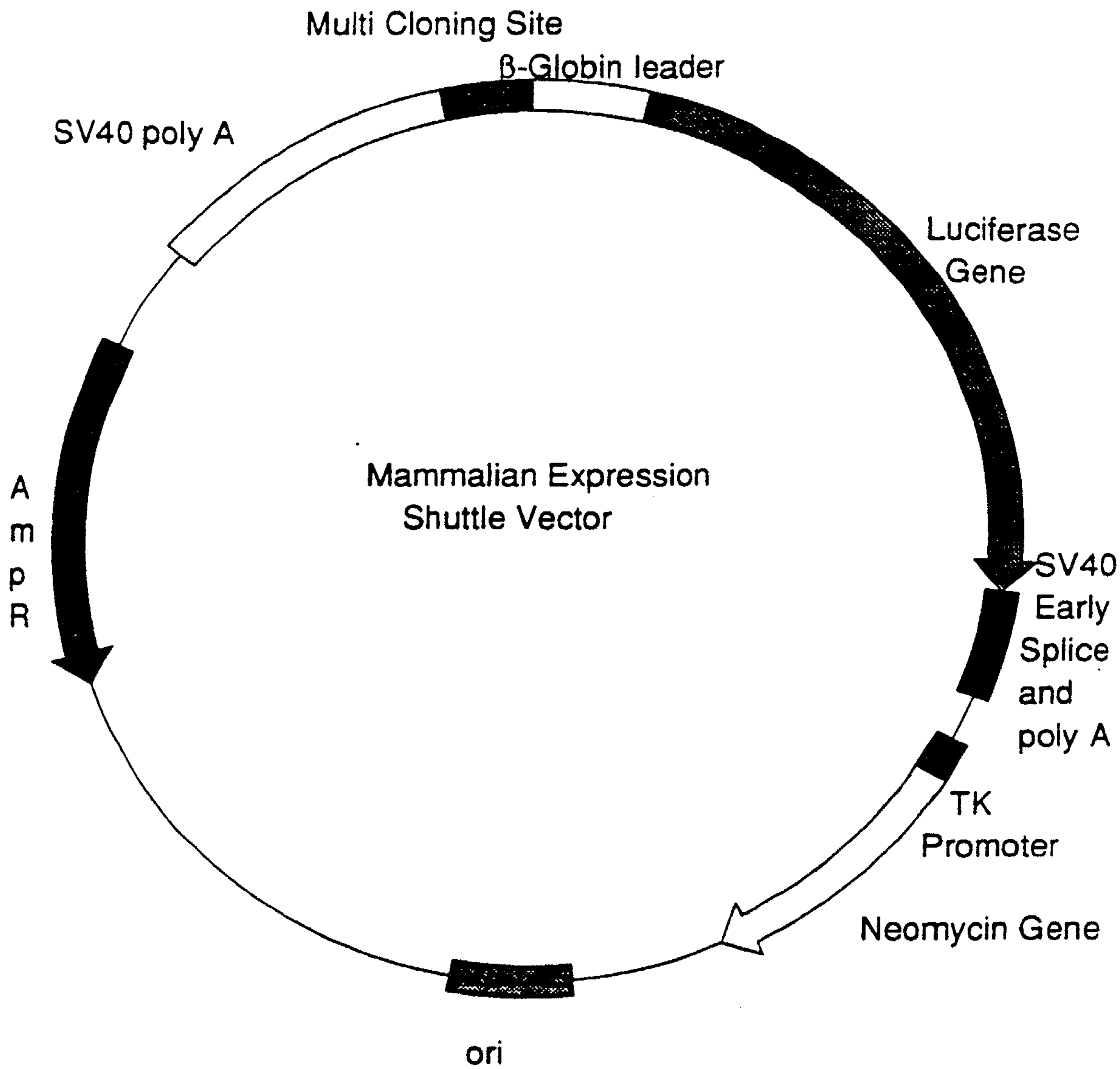


Figure 2.
pDO432.

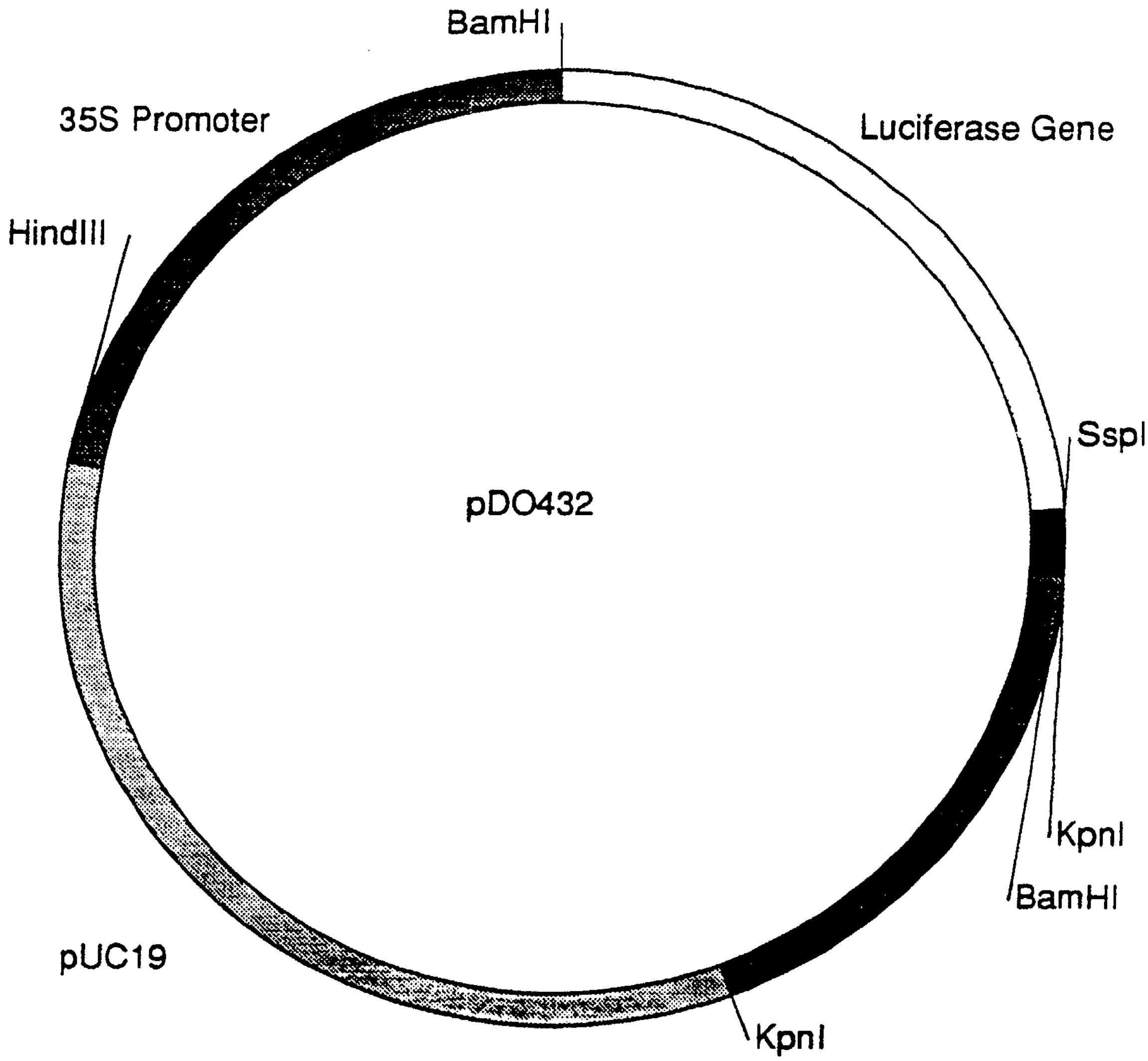


Figure 3.
pSVLuci.

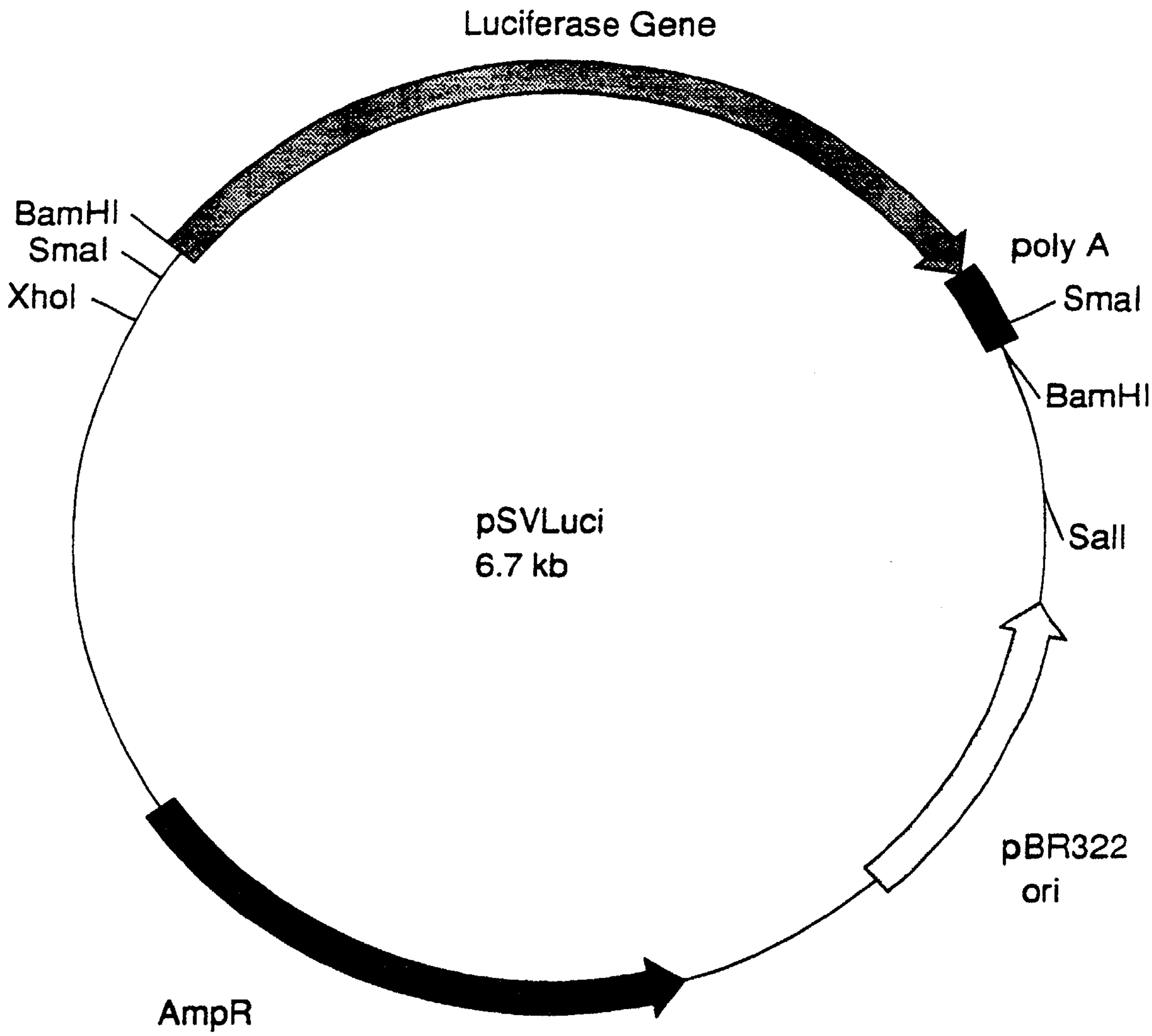


Figure 4.
pMLuci.

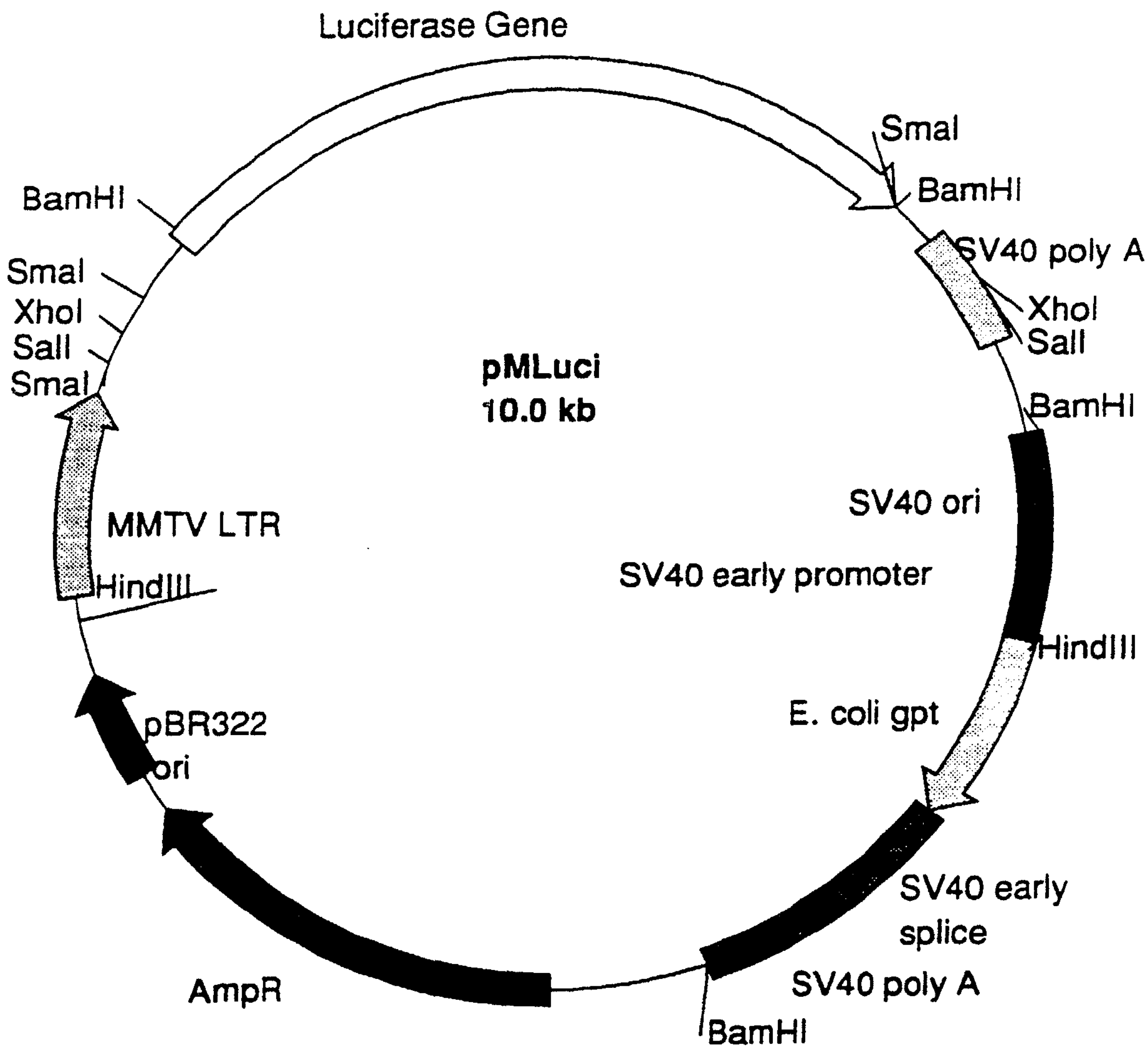


Figure 5.
Sequence of the pUV oligonucleotides.

pUV1:
5'TCGACCCGGGCGGCCGCTGATCAGACGTCGGGCC[illegible]GGTACCGTGCACTACGTAAGATCTAA
GCTT3'

pUV2:
5'ACTAGTCTGCAGGCTAGCACTCTTCTGGTCCCCACAGACTCAGAGAGAACCCACCATGGA
3'

pUV3:
5'AGACGCCAAAAACATCAAGAAAGGCCCGGCGCCATTCTATCCTCTAGAGGGGATCCAGC
TG3'

pUV4:
5'TAGATCTTACGTAGTGCACGGTACCGGGCCCGACGTCTGATCAGCGGCCGCCCGGG3'

pUV5:
5'GGTGGGTTCTCTCTGAGTCTGTGGGGACCAGAAGAGTGCTAGCCTGCGACTAGTAAGCT3'

pUV6:
5'AATTCAGCTGGATCCCCTCTAGAGGATAGAATGGCGCCGGGCCTTTCTTGATGTTTTTGGCGT
CTTCCAT3'

Figure 6

Construction of pUV001

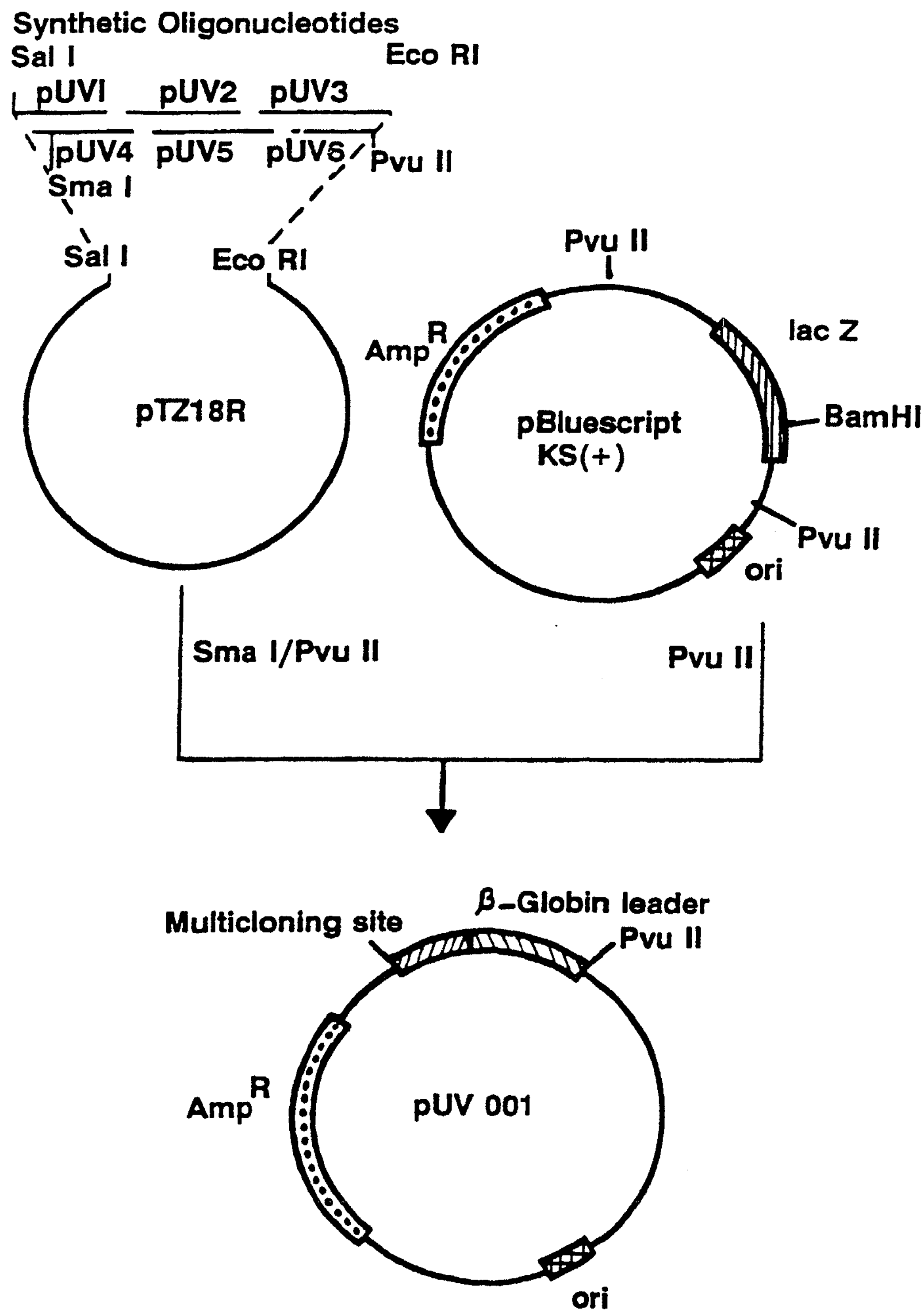


Figure 7
Construction of pUV100

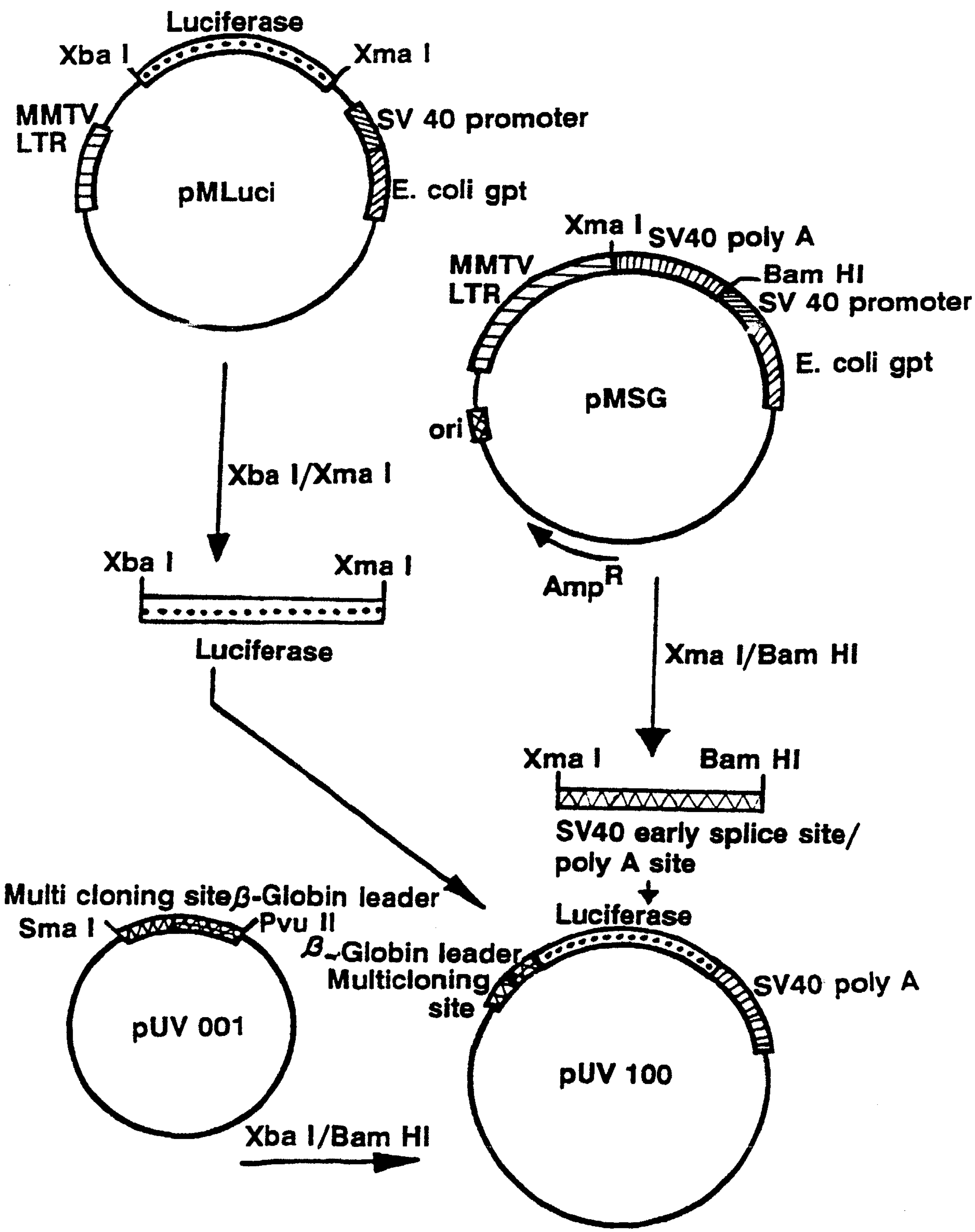


Figure 8
Construction of pUV100-3

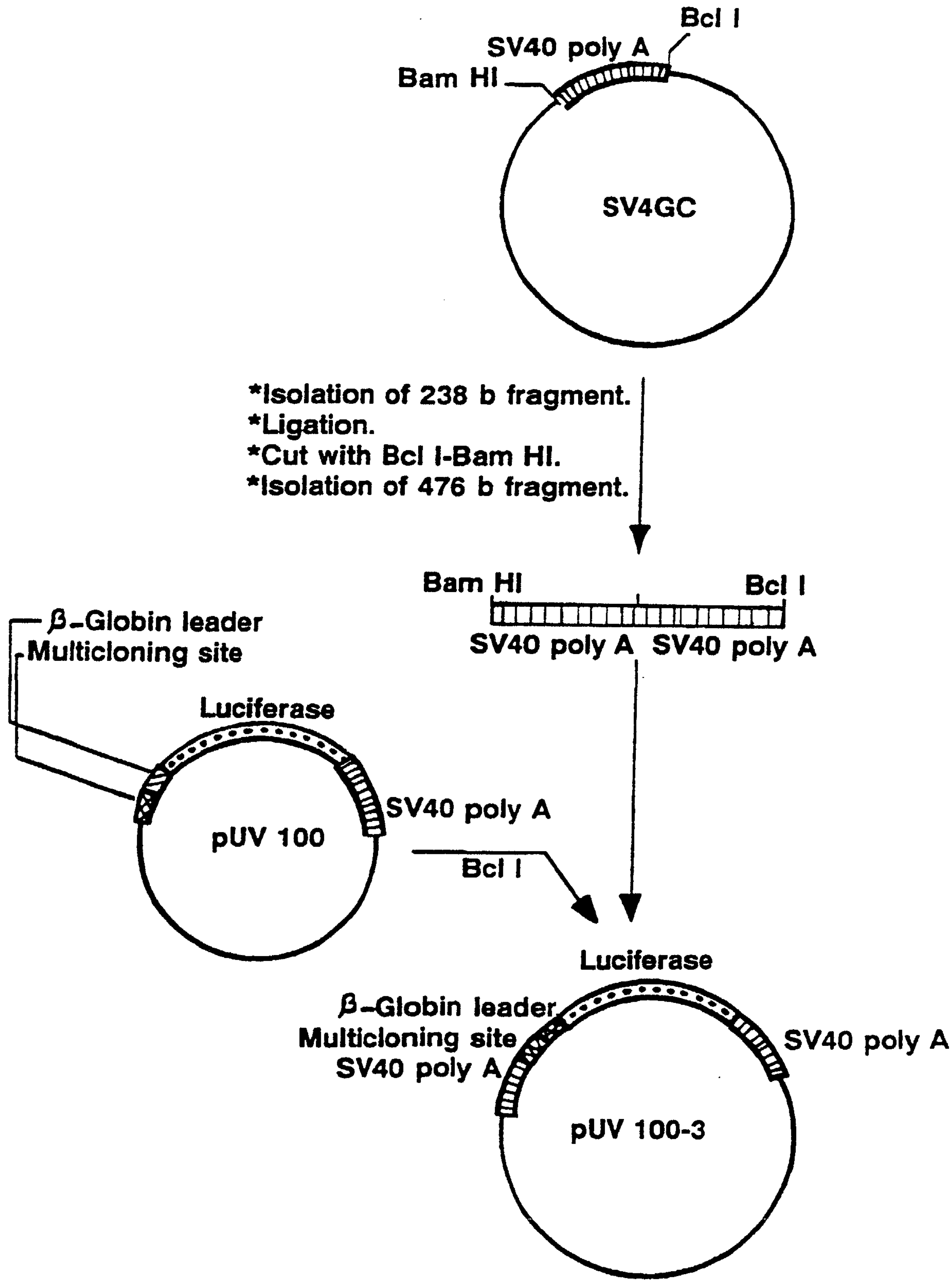


Figure 9

Construction of pUV102 and pUV103

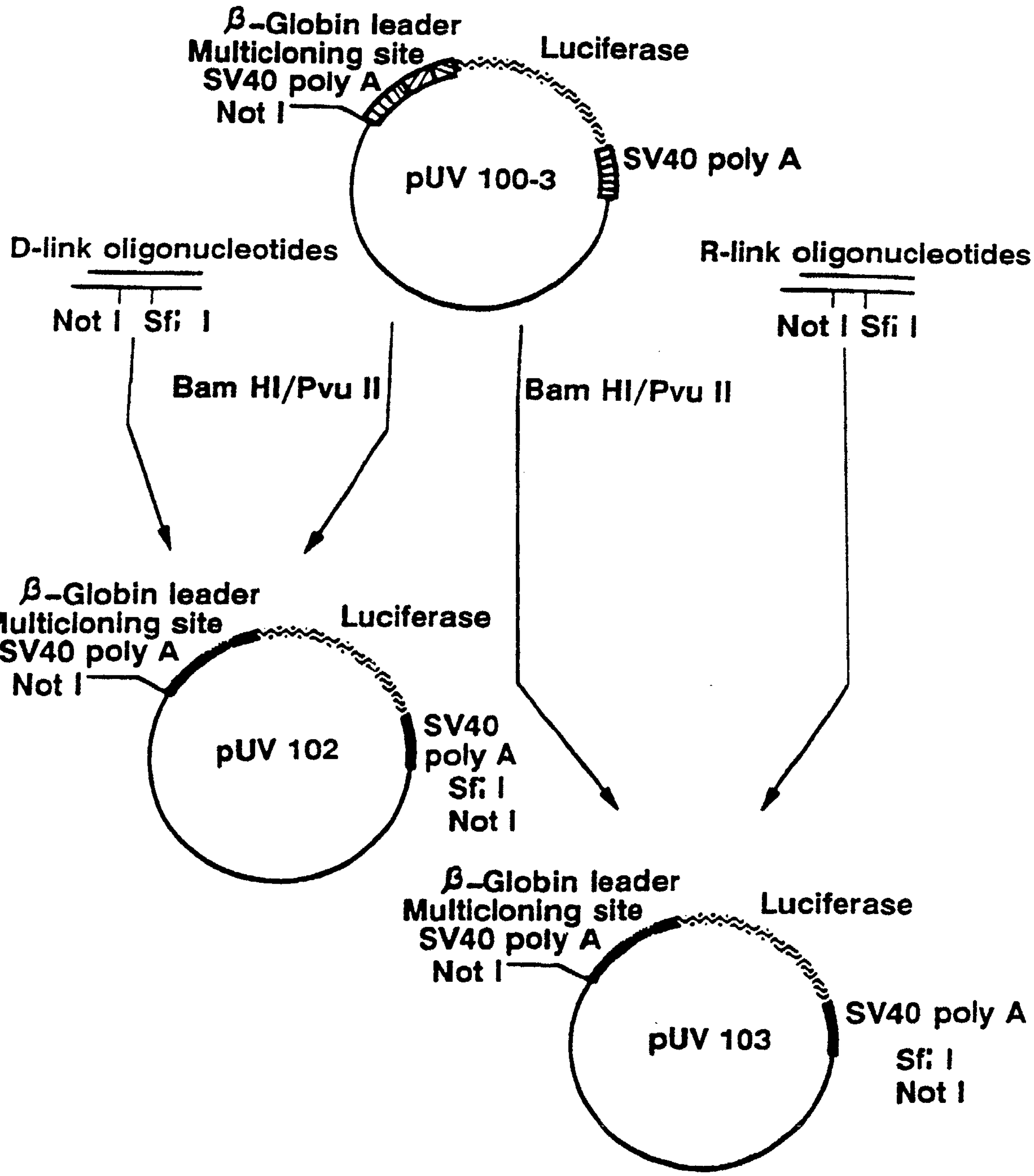


Figure 10

Synthetic HSV-TK Promoter

Oligo #1: 5'-AGCTTGGCCCCTAGGGCCACTAGTCTGCAGCTATGATGACACAA
ACCCCGCCCAGCGTCTTGTCATTGGCGA-3'

Oligo #2: 3'-ACCGGGGATCCCGGTGATCAGACTCGATACTACTGTGTTTGGGG
CGGGTCGCAGAACAGTAACCGCTTAAGCT-5'

Oligo #3: 5'-ATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCGAGGTC
CACTTCGCATATTAAGGTGACGCGTGTGGG-3'

Oligo #4: 3'-TGTGCGTCTACGTCAGCCCCGCCGCGCCAGGCTCCAGGTGAAG
CGTATAATTCCACTGCGCACACCCGATC-5'

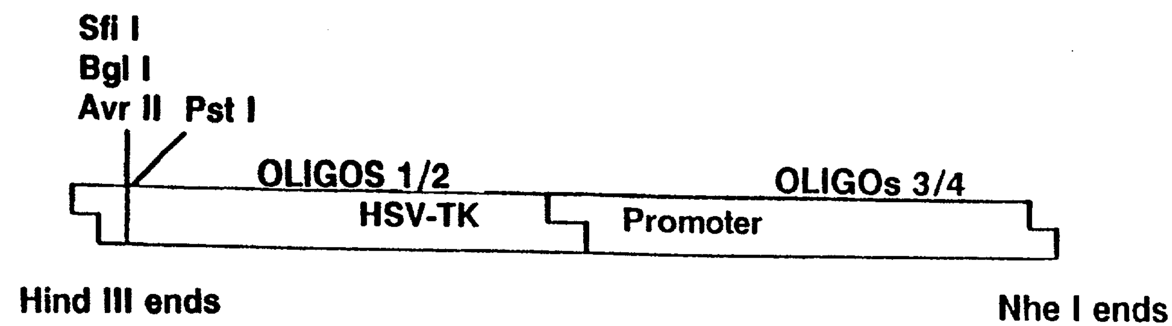


Figure 11

Constuction of pTKL100

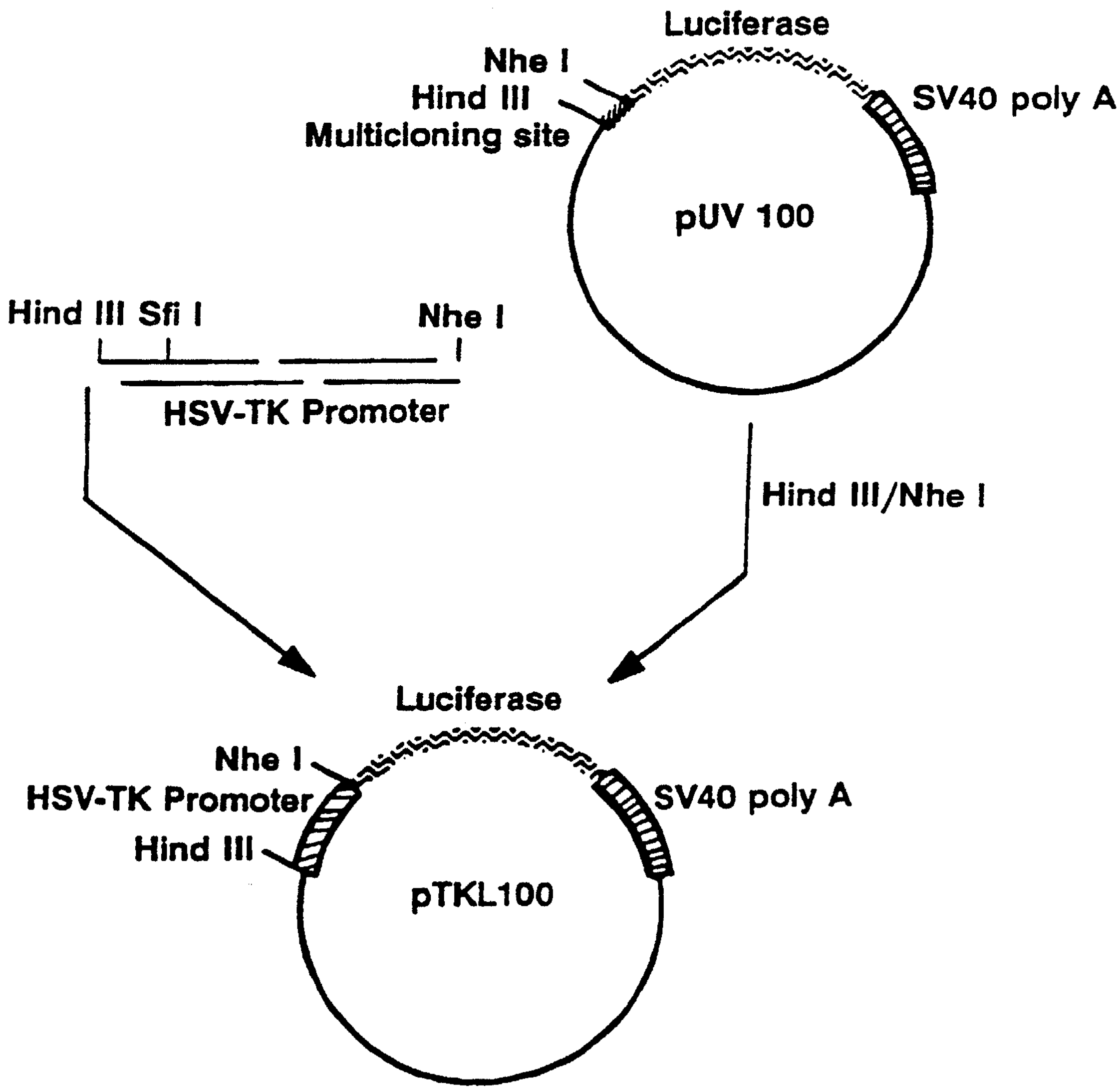


Figure 12

Construction of pTKNEO

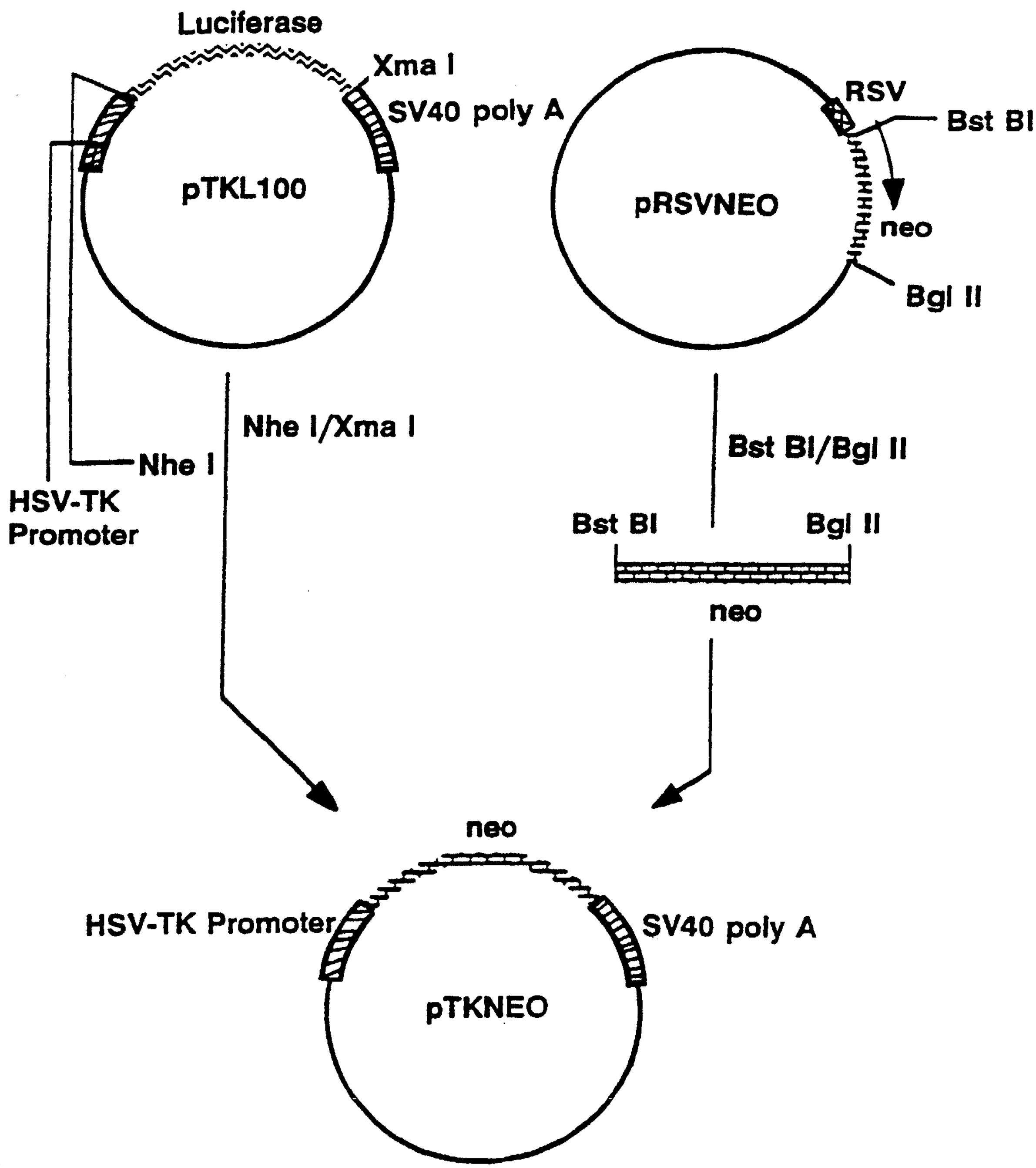


Figure 13

Construction of pTKNEO2

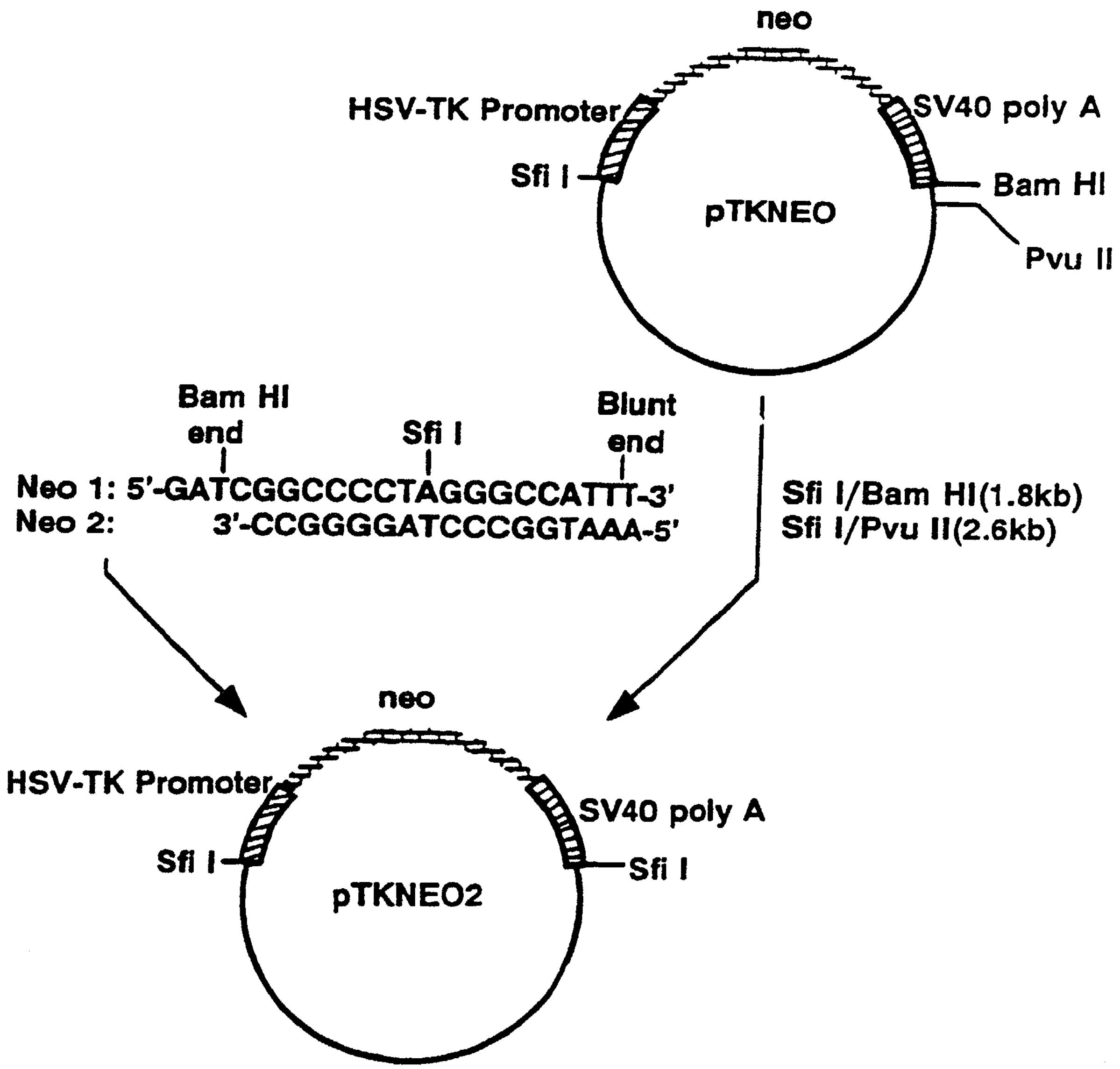


Figure 14

Construction of pTKNEO3

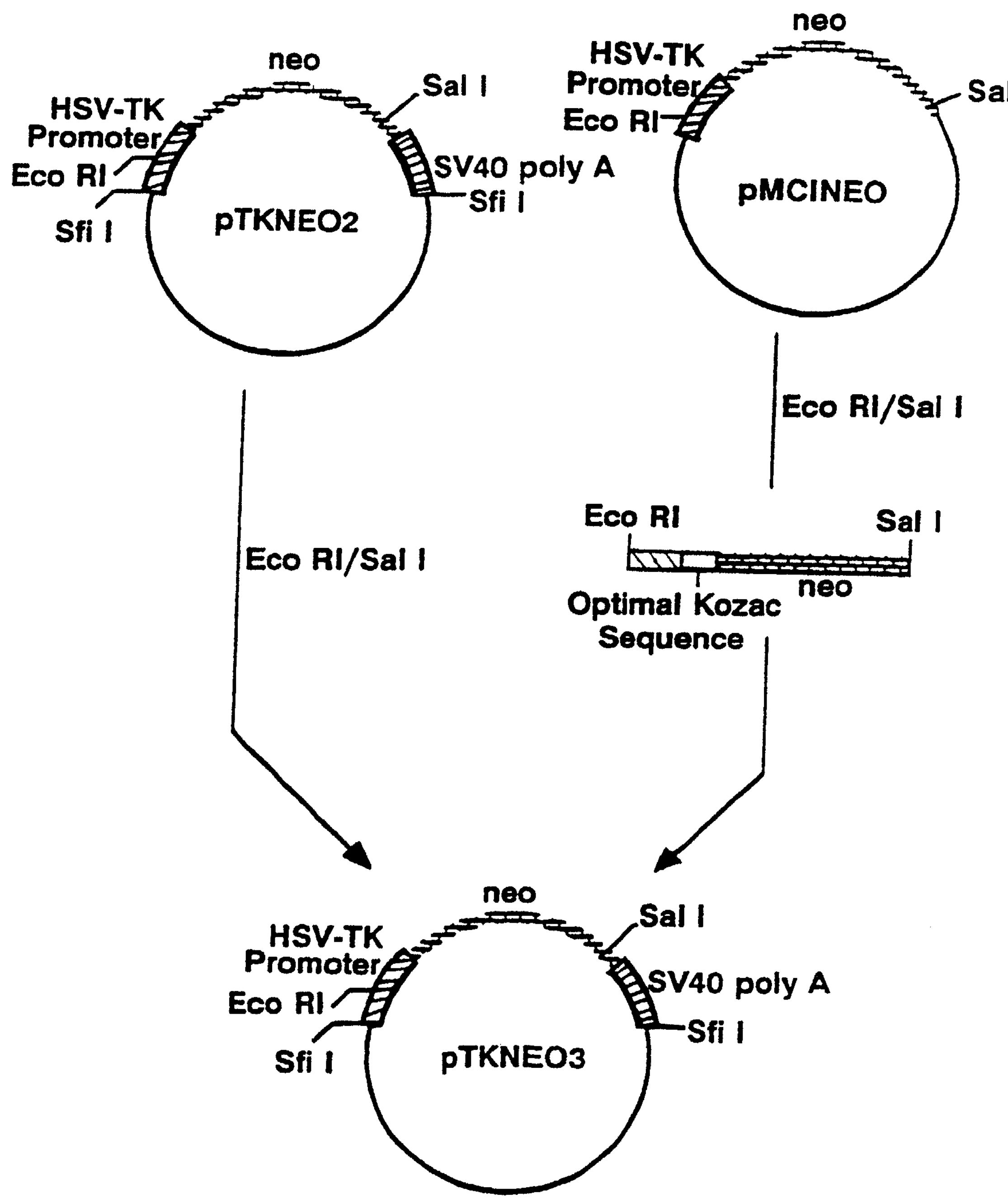


Figure 15 pUXLuci

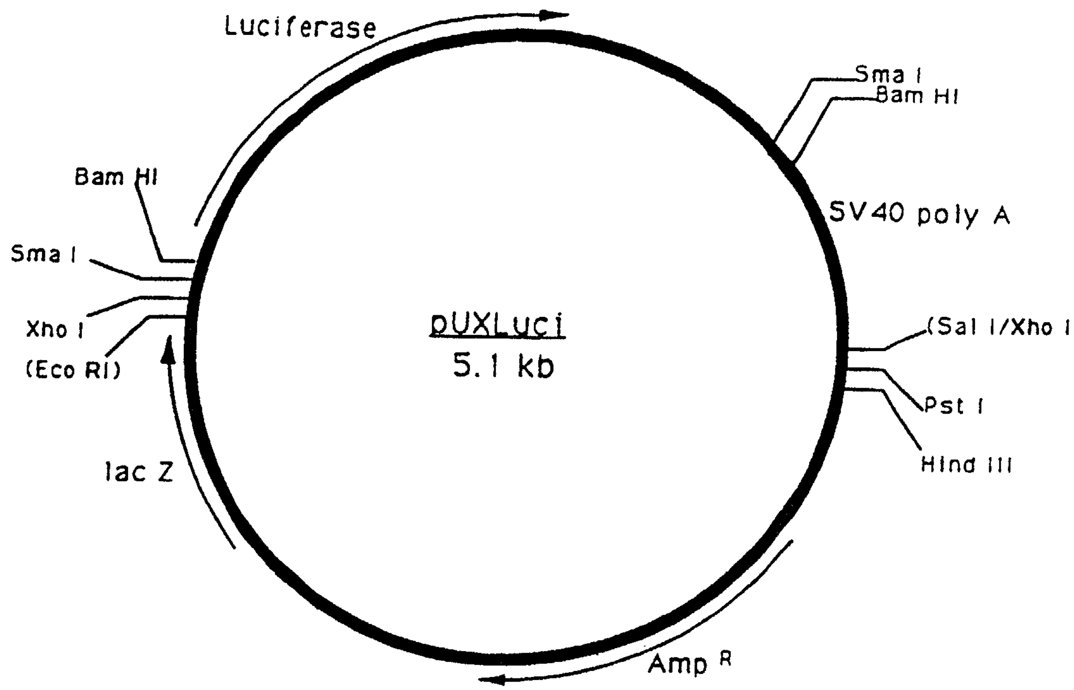


Figure 16 phGH - CAT

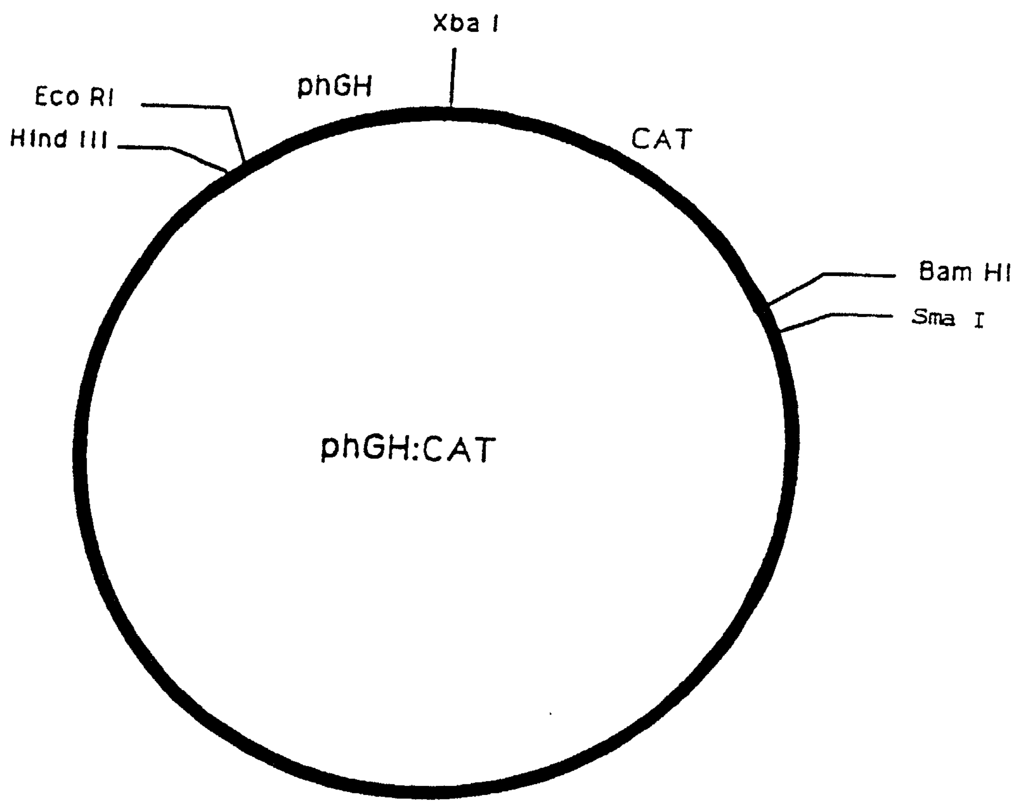
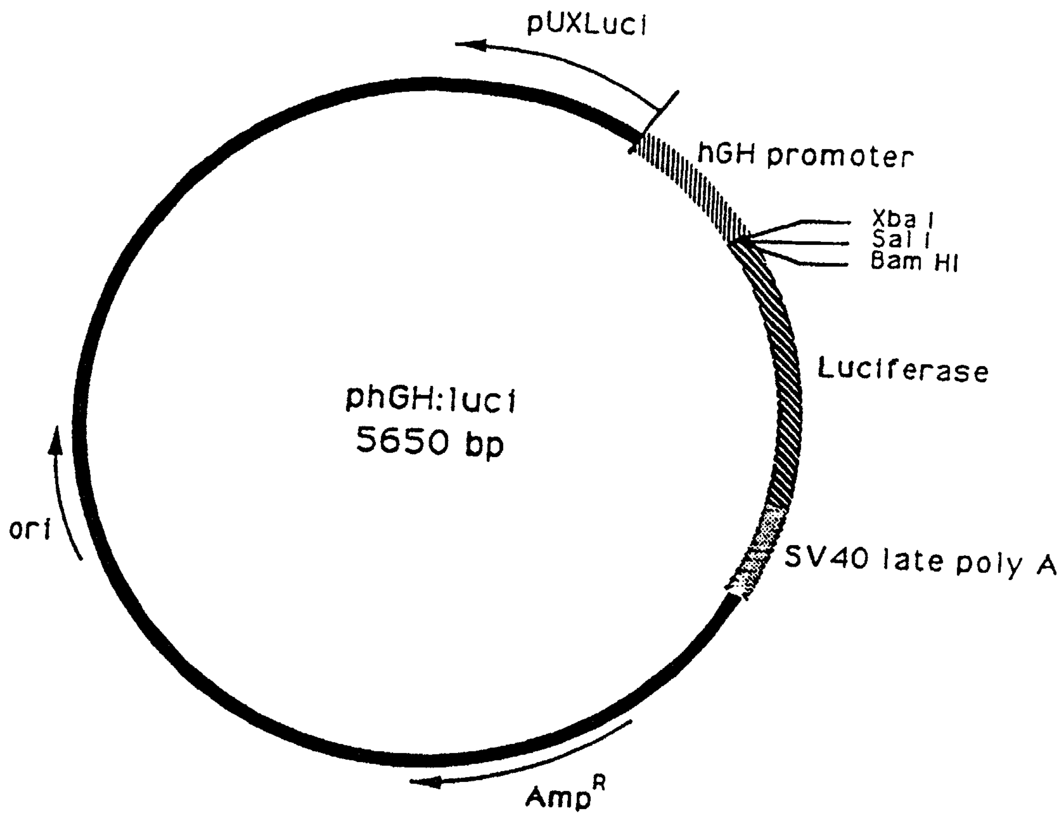


Figure 17 phGH - Luci



The Structure of pNEU106

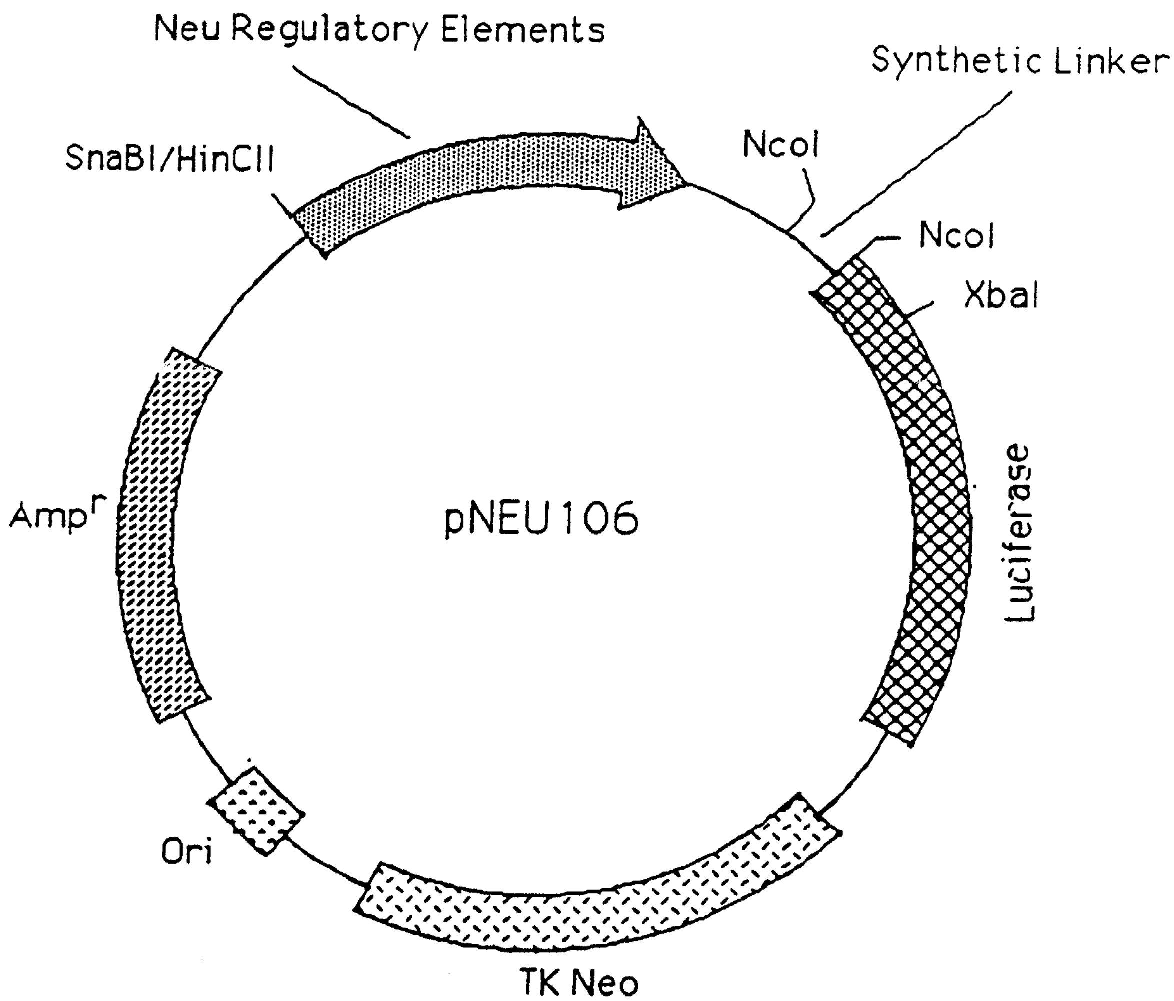


Figure 19

The Structure of pKRAS106

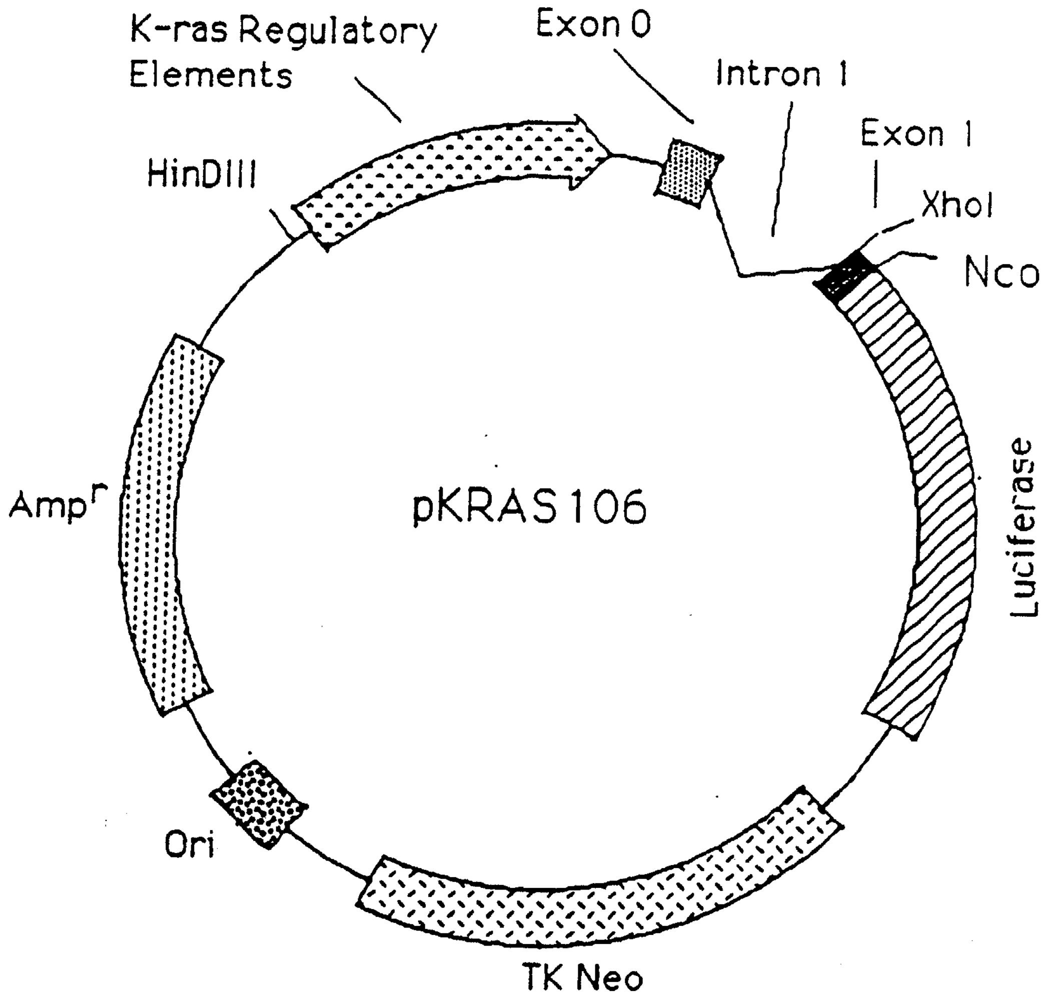


Figure 20

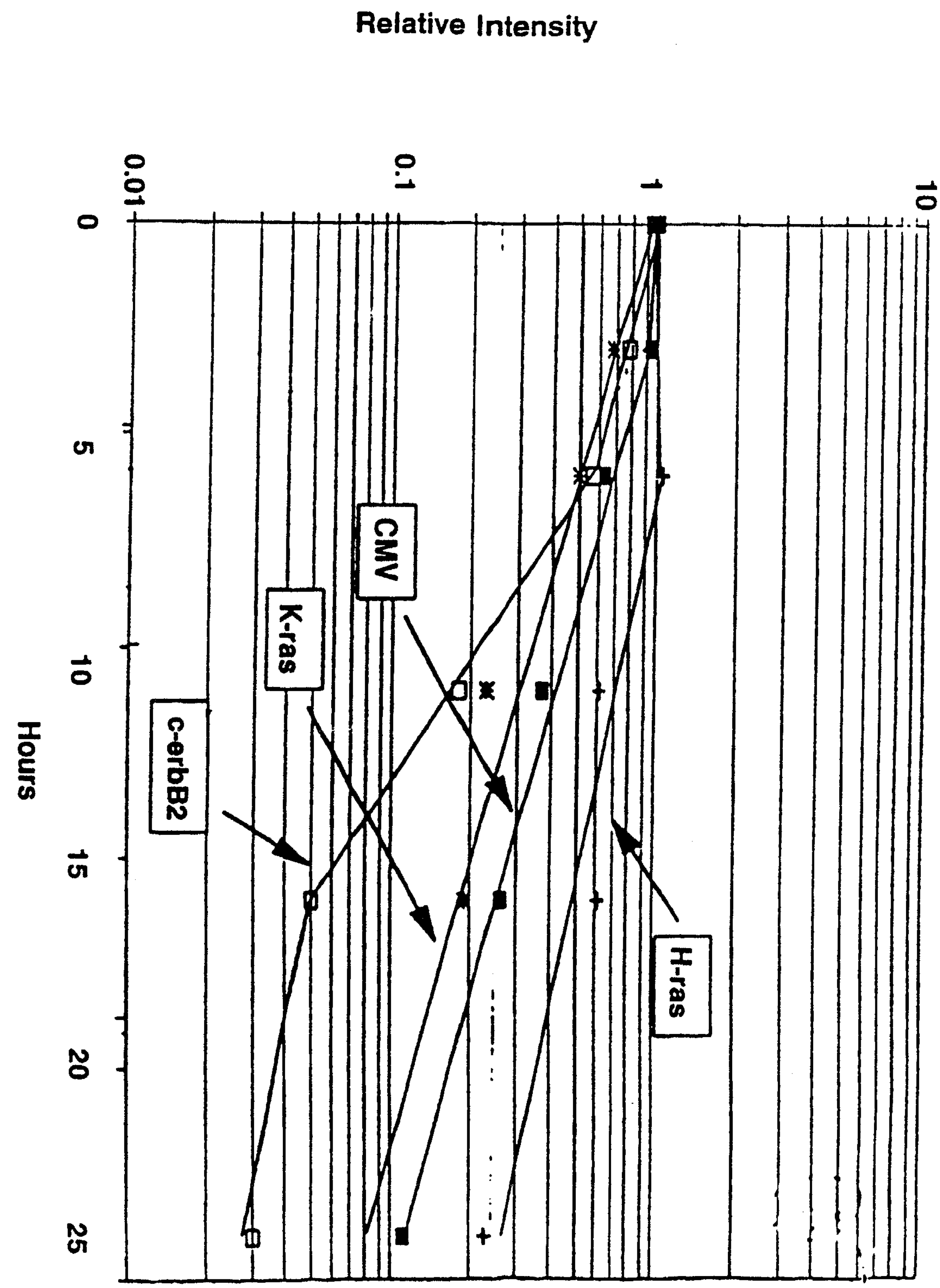

Relative Intensity
10
1
0.1
0.01
0
5
10
15
20
25
Hours
CMV
K-ras
c-erbB2
H-ras

Figure 21

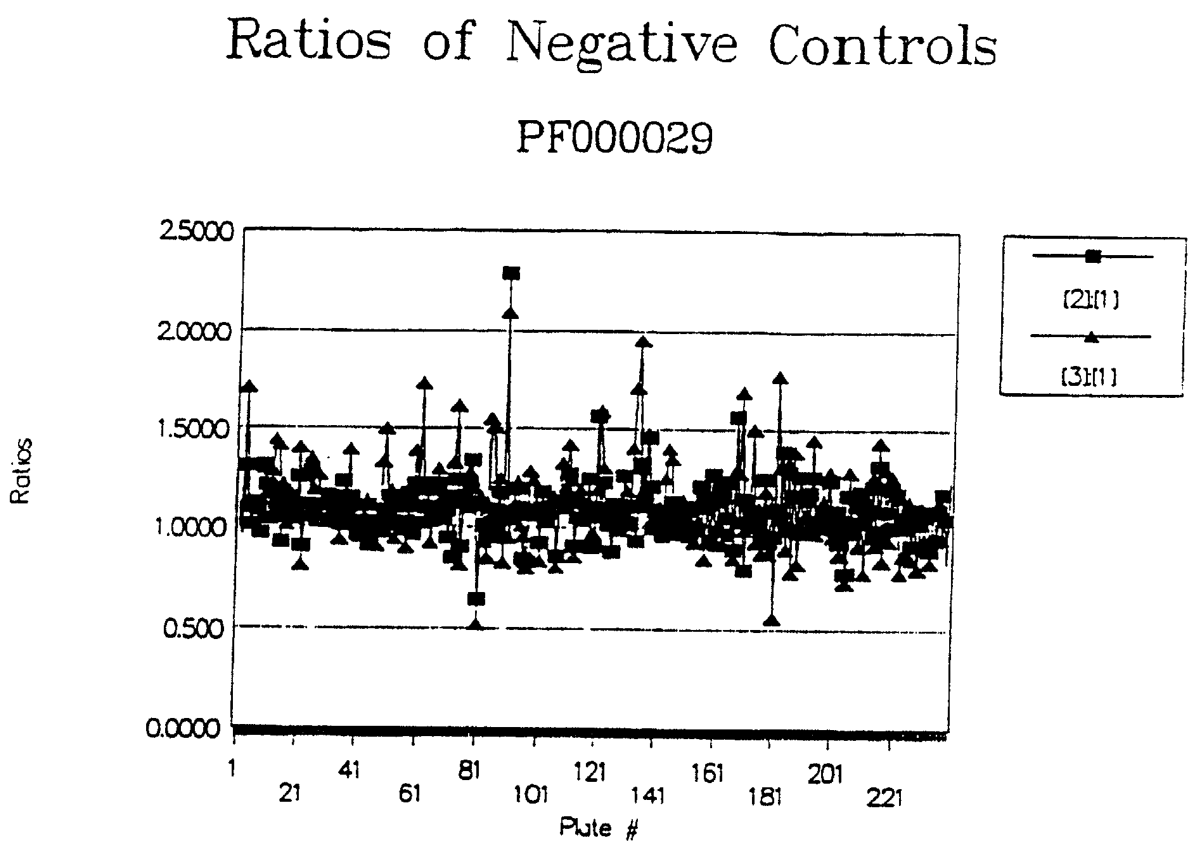

Ratios of Negative Controls
PF000029
2.5000
2.0000
1.5000
1.0000
0.500
0.0000
Ratios
1
21
41
61
81
101
121
141
161
181
201
221
Plate #
(2):(1)
(3):(1)

Figure 22

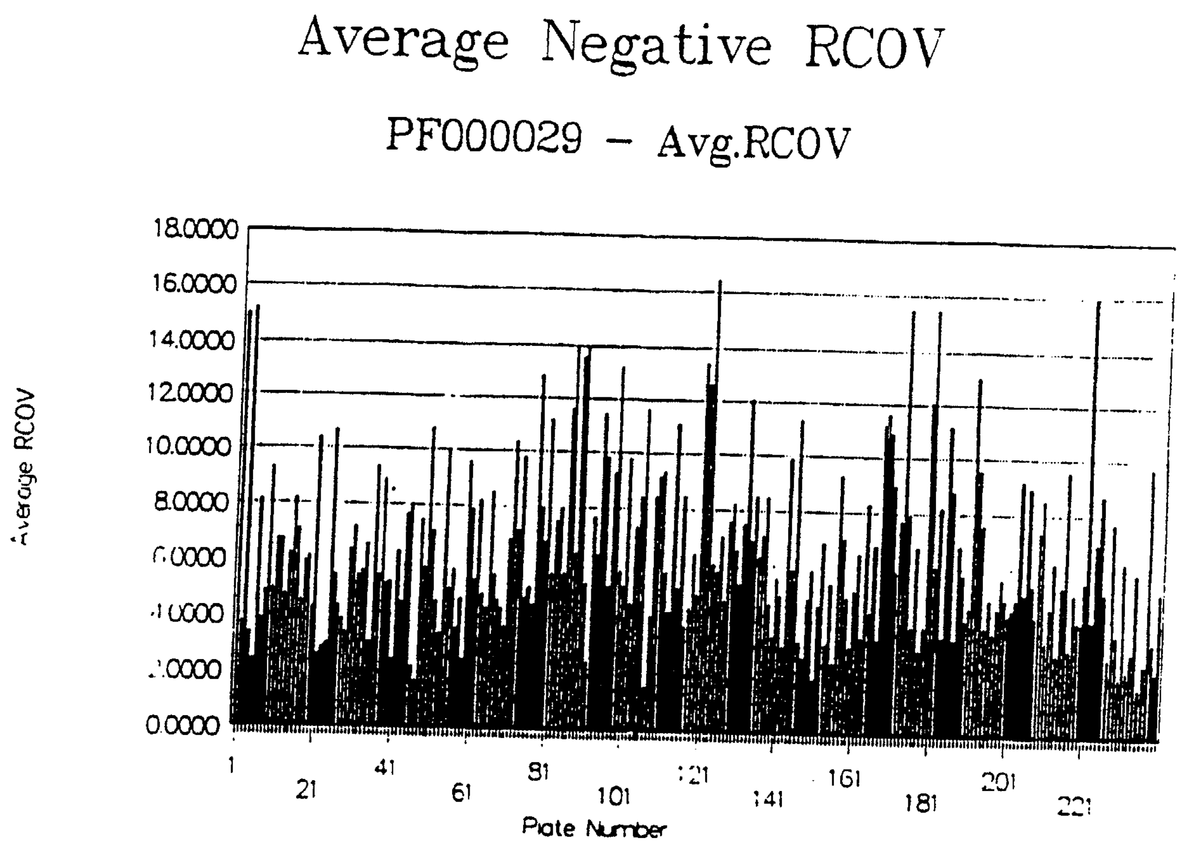

Average Negative RCOV
PF000029 – Avg.RCOV
18.0000
16.0000
14.0000
12.0000
10.0000
8.0000
0.0000
Average RCOV
1
21
41
61
81
101
141
161
181
201
Plate Number

Figure 23

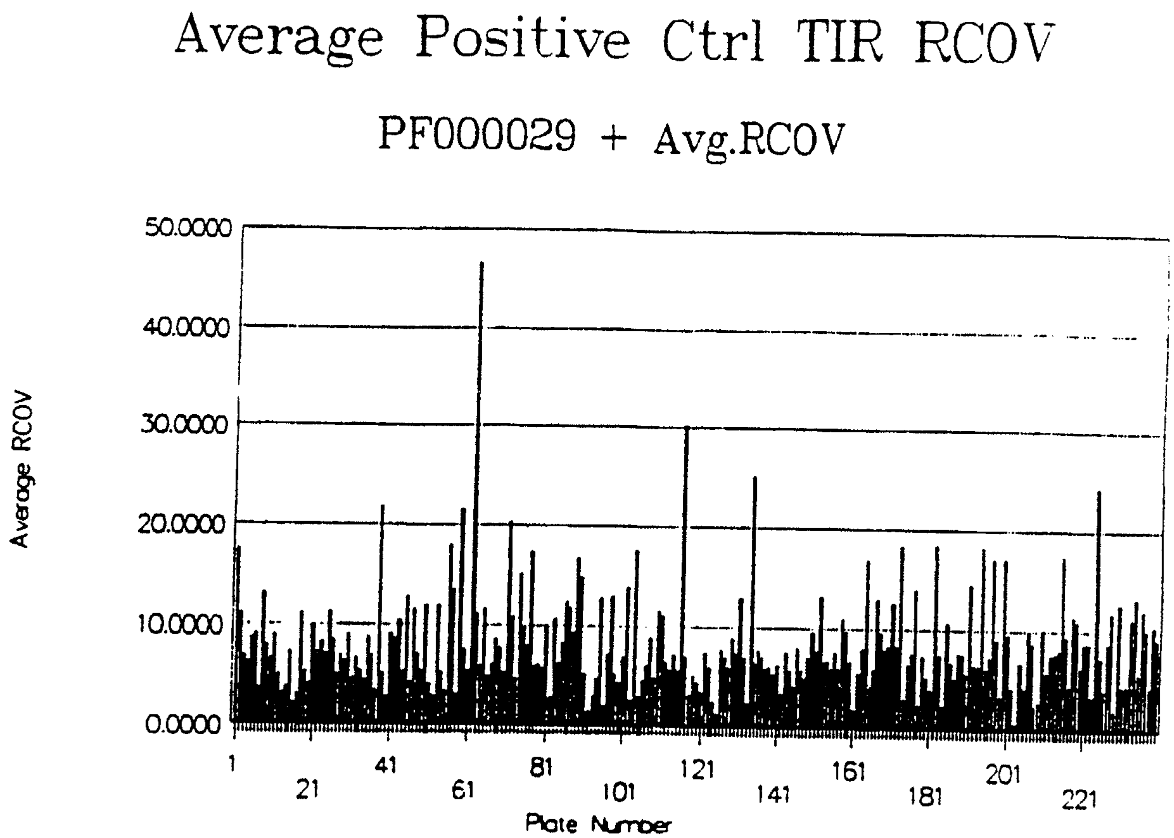
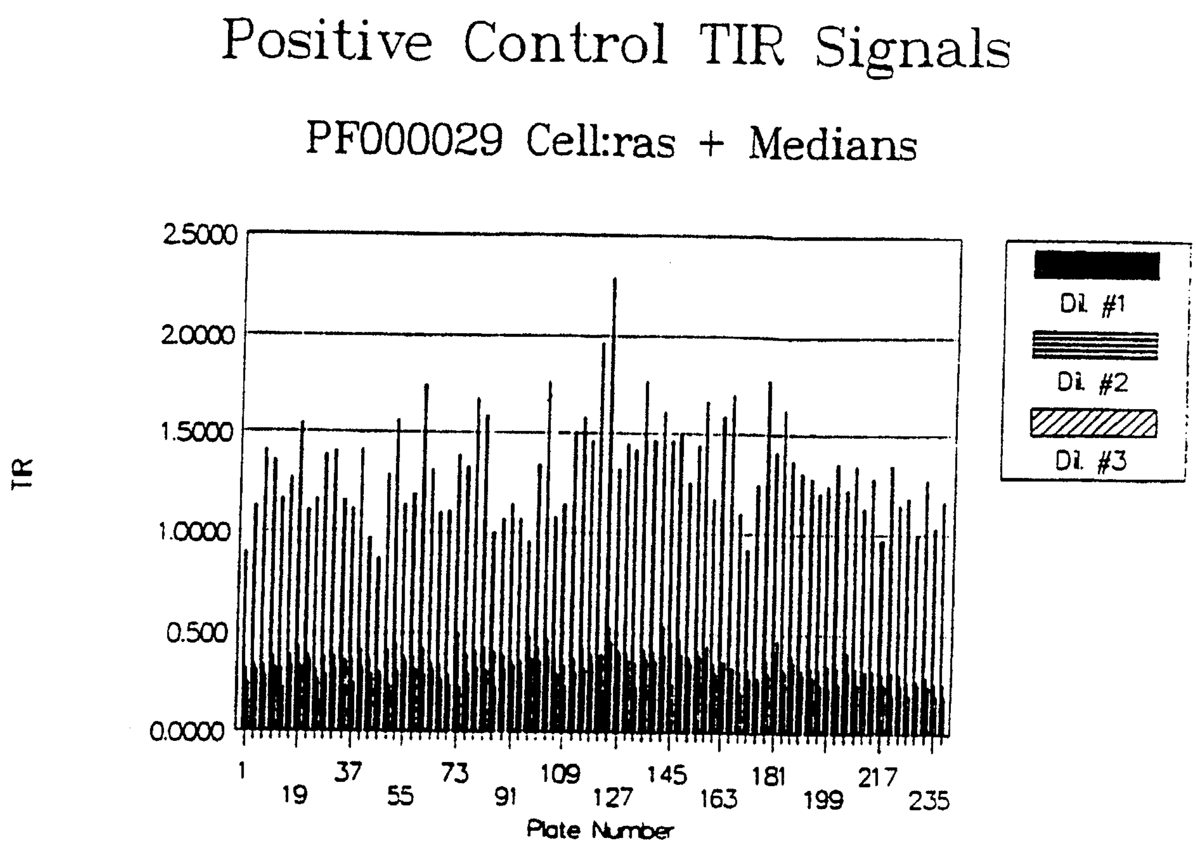

Average Positive Ctrl TIR RCOV
PF000029 + Avg.RCOV
Average RCOV
50.0000
40.0000
30.0000
20.0000
10.0000
0.0000
1
21
41
61
81
101
121
141
161
181
201
221
Plate Number

Figure 24


Positive Control TIR Signals
PF000029 Cell:ras + Medians
2.5000
2.0000
1.5000
1.0000
0.500
0.0000
TIR
1
19
37
55
73
91
109
127
145
163
181
199
217
235
Plate Number
Di. #1
Di. #2
Di. #3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00419

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): Please See Attached Sheet.
US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6, 7.1, 69.1, 69.4, 69.52, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,738,922 (Haseltine et al.) 19 April 1988. See col 1-10 and the examples.	1-66
Y	US, A, 4,740,461 (Kaufman) 26 April 1988. See col 1-16 and the examples.	1-66
Y	EP, A, 0,117,058 (Levinson et al.) 29 August 1984. See at least the abstract and pages 5-18.	1-66
Y	Mol. Cell. Biol. volume 7, no. 6, issued June 1987, Angel et al., "12-O-Tetradecanoyl-Phorbol-13-Acetate Induction Of The Human Collagenase Gene Is Mediated By An Inducible Enhancer Element Located In The 5'-Flanking Region", pages 2256-2266. See the abstract and the figures.	1-66
Y	Proc. Natl. Acad. Sci. USA. Volume 83, issued May 1986, Kaushansky et al., "Genomic Cloning, Characterization, Multilineage Growth Promoting Activity Of Human Granulocyte-Macrophage Colony-Stimulating Factor", pages 3101-3105. See figure 2, and page 3105.	1, 3-14, 17-42

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
04 MAY 1992	18 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	Christopher Low

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EMBO J. Volume 6, No. 9, issued September 1987, Ladner et al., "Human CSF-1: Gene Structure And Alternative Splicing Of mRNA", pages 2693-2698. See at least pages 2693, 2696-2997.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y	EMBO J. Volume 6, No. 4, issued April 1987, Lefevre et al., "Tissue-Specific Expression Of The Human Growth Hormone Gene Is Conferred In Part By The Binding Of A Specific Trans-Acting Factor", pages 971-981. See at least page 971.	1, 3-14, 17, 19, 21, 67-69, 73-109

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice)
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Proc. Natl. Acad. Sci. USA. Volume 82, issued November 1985, Lin et al., "Cloning And Expression Of The Human Erythropoietin Gene", pages 7580-7584. See entire document.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y	EMBO J. Volume 5, No. 3, issued March 1986, Nagata et al., "The Chromosomal Gene Structure And Two mRNAs For Human Granulocyte Colony-Stimulating Factor", pages 575-581. See at least the abstract.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y	Cell Volume 47, issued 10 October 1986, Yang et al., "Human IL-3 (Multi-CSF): Identification By Expression Cloning Of A Novel Hematopoietic Growth Factor Related To Murine IL-3", pages 3-10. See figure 4, and page 6.	1, 3-14, 17, 19, 35, 67-69, 73-109
Y	de Serres et al., "Chemical Mutagens. Principles And Methods For Their Detection", published 1980 by Plenum Press (New York), pages 331, and 365-473. See at least pages 331, 367-369, 377.	1-23, 58-79
Y	Science Volume 227, issued 15 March 1985, Engebrecht et al., "Measuring Gene Expression With Light", pages 1345-1347. See entire document.	1-135
Y	Science Volume 236, issued 05 June 1987, Maniatis et al., "Regulation Of Inducible Tissue-Specific Gene Expression", pages 1237-1245. See pages 1237, 1239, 1240, 1243.	1-23, 58-79
Y	Molec. Cell. Biol. Volume 7, No. 2, issued February 1987, de Wet et al., "Firefly Luciferase Gene: Structure And Expression In Mammalian Cells", pages 725-737. See the abstract, figures 1 and 3, pages 729-734.	1-109
Y	Exp. Hematol., vol. 16, issued 1988, Bickel et al., "Granulocyte-Macrophage Colony-Stimulating Factor Regulation In Murine T Cells And Its Relation To Cyclosporin A", pages 691-695. See entire document.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y	US, A, 4,601,978 (Karin) 22 July 1986, see entire document.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y	WO, A, 89/02472 (Shannon et al.) 23 March 1989. See entire document.	1-23, 58-79
Y	Cell, vol. 49, issued 19 June 1987, Angel et al., "Phorbol Ester-Inducible Genes Contain A Common Cis Element Recognized By A TPA-Modulated Trans-Acting Factor", pages 729-739. See entire document.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y	Science, vol. 230, issued 18 October 1985, Kawasaki et al., "Molecular Cloning Of A Complementary DNA Encoding Human Macrophage-Specific Colony-Stimulating Factor (CSF-1)", pages 291-296, see entire document.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y	Proc. Natl. Acad. Sci. USA., vol. 81, issued August 1984, Kronke et al., "Cyclosporin A Inhibits T-Cell Growth Factor Gene Expression At The Level Of mRNA Transcription", pages 5214-5218. See entire document.	1, 3-14, 17, 19, 21, 67-69, 73-109

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Gene Amplification (Schimke, R. T., ed.), issued 1982, Mayo et al., "Altered Regulation Of The Mouse Metallothionein I Gene Following Gene Amplification Or Transfection", pages 67-73. See entire document.	1, 3-14, 17, 19, 21, 67-69, 73-109
Y,P	US, A, 5,070,012 (Nolan et al.) 03 December 1991. See entire document.	1-135
Y	US, A, 4,981,783 (Augenlicht) 01 January 1991 see entire document.	1-135
Y	US, A, 4,806,463 (Goodchild et al.) issued 21 February 1989. See entire document.	110, 112-135
Y	US, A, 4,861,709 (Ulitzur et al.), 29 August 1989. See entire document.	24-41, 52-57
Y	US, A, 4,935,363 (Brown et al.), 19 June 1990. See entire document.	1-135
Y	Nature, vol. 346, issued 16 August 1990, Standaert et al., "Molecular Cloning And Overexpression Of The Human FK506-Binding Protein FKBP", pages 671-674. See entire document.	1-135
Y	J. Immunol., vol. 143, issued 15 July 1989, Tocci et al., "The Immunosupprressant FK506 Selectively Inhibits Expression Of Early T Cell Activation Genes", pages 718-726. See entire document.	1-135
Y	Bio/Technol., vol. 7, issued March 1989, Ratner, "Can The Antisense Message Be Delivered?", page 207. See entire document.	18-20, 111-135
Y	Bio/Techniques, vol. 7, no. 6, issued June 1989, Cao et al., "A Simple And Inexpensive System To Amplify DNA By PCR", pages 566-567. See entire document.	67-109
Y	Bio/Techniques, vol. 7, no. 6, issued June 1989, Lim et al., "A Simple Assay For DNA Transfection By Incubation Of The Cells In Culture Dishes With Substrates For Beta-Galactosidase", pages 576-579. See entire document.	67-109
Y	Bio/Technol., vol. 7, issued March 1989, McCall et al., "Biotherapy: A New Dimension In Cancer Treatment", pages 231-240. See entire document.	111-135
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Brenner et al., "Message Amplification Phenotyping (MAPPing): A Technique To Simultaneously Measure Multiple mRNAs From Small Numbers Of Cells", pages 1096-1103. See entire document.	67-109
Y	Bio/Techniques, vol. 7, issued November/December 1989, Munjaal et al., "In Situ Detection Of Progesterone Receptor mRNA In The Chicken Oviduct Using Probe-On Slides", pages 1104-1108. See entire document.	1-135

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Brasier et al., "Optimized Use Of The Firefly Luciferase Assay As A Reporter Gene In Mammalian Cell Lines", pages 1116-1122. See entire document.	67-109
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Slack et al., "Application Of The Multiscreen System To Cytokine Radioreceptor Assays", pages 1132-1138. See entire document.	67-109
Y	Bio/Techniques, vol. 8, no. 1, issued January 1990, Rao et al., "A Quantitative Assay For ß-D-Glucuronidase (GUS) Using Microtiter Plates", pages 38-40. See entire document.	67-109
Y	Bio/Techniques, vol. 8, no. 3, issued March 1990, Willingham et al., "A Reversible Multi-Well Chamber For Incubation Of Cultured Cells With Small Volumes: Application To Screening Of Hybridoma Fusions Using Immunofluorescence Microscopy", pages 320-324. See entire document.	67-109
Y	Bio/Techniques, vol. 9, no. 4, issued October 1990, Pons et al., "A New Cellular Model Of Response To Estrogens: A Bioluminescent Test To Characterize (Anti)Estrogen Molecules", pages 450-459. See entire document.	1-135
Y	US, A, 4,761,371 (Bell et al.) 02 August 1988 See entire document.	2-12, 15-20, 43-66, 70-135

nternational Application No. PCT/US92/08419

URTHER INFORMATION CONTINUED FROM THE PREVIOUS SHEET
(Not for publication)

CLASSIFICATION OF SUBJECT MATTER: IPC (5):
C12P
P 21/00, 21/02; C12N 5/00, 7/04, 15/00, 1/38; C12Q 1/66, 1/68, 1/00

CLASSIFICATION OF SUBJECT MATTER: US CL :

/6, 7.1, 69.1, 69.4, 69.52, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

FIELDS SEARCHED
er Documents Searched:

TO APS - USPAT, JPOABSDIALOG - BIOSIS, CHINESE PATENT ABSTRACTS, CLAIMS/USPATENTS, INPADOC/FA
.Y AND
AL STATUS, WORLD PATENT ABSTRACTS

rch Terms
mammal, toxicity, toxic, chemical?, carcinogen?, oncogen?, transcript?, growth factor, r
ptor, porcine, human, fish,
transforming, insulin, somatomedin, platelet derived,
endothelial, fibroblast, nerve, bone morphogenic protein,
granulocyte, macrophage, colony, stimulat?, erythropoietin,
antisense, cytokine?, interleukin?.

OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1, 3-12, 13, 14, 17-42, 110, 112-135, drawn to a methodfor transcriptional modul
on of DNA encoding a growth factor
are classified in Class 435, subclasses 69.4, 69.52, and 70.1.

II. Claims 2-12, 15-20, 43-66, 111-135, drawn to a method for
transcriptional modulation of DNA encoding a growth factorreceptor are classified in Cl
435, subclasses 69.1 and 70.1.

III. Claims 67, 68, 73-109, drawn to a method for testing a molecule
for effecting transcription of a growth factor DNA by detecting
the signal from a polypeptide are classified in Class 435,subclass 7.1.

IV. Claims 69, 73-92, 103-109, drawn to a method for testing a
molecule for effecting transcription of a growth factor DNA by
detecting the signal from the mRNA produced are classified inClass 435, subclass 6.

V. Claims 70, 71, 73-109, drawn to a method for testing a molecule
for effecting transcription of a growth factor receptor DNA by
detecting the signal from a polypeptide are classified in Class435, subclass 7.1.

VI. Claims 72-92, 103-109, drawn to a method for testing a molecule
for effecting transcription of a growth factor receptor DNA by
detecting the signal from the mRNA produced are classified inClass 435, subclass 6.